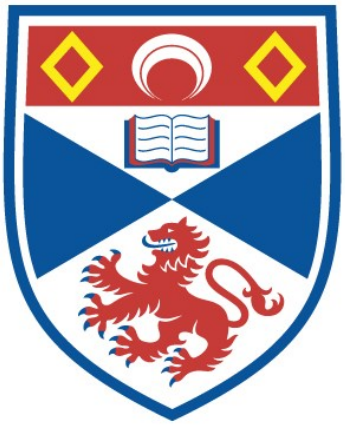
CELLULAR AND ENVIRONMENTAL CONTROL OF GLYCINE TRANSPORT IN HELA AND XENOPUS LAEVIS CULTURED CELLS

Susan Patricia Hume

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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Cellular and environmental control of glycine transport in HeLa and Xenopus laevis cultured cells

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by

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ACADEMIC RECORD

I first matriculated at the University of St Andrews in October 1967. and graduated with the degree of B.Sc. Hons. (1st Class) in Physiology in June 1971. I matriculated as a research student in the Department of Physiology, University of St Andrews in October 1971.

CERTIFICATE

I hereby certify that Susan P. Hume has spent nine terms engaged in research work under my direction, and that she has fulfilled the conditions of General Ordinance No.12 (Resolution of the University Court No.1 1967), and that she is qualified to submit the accompanying thesis for the Degree of Doctor of Philosophy.

SUMMARY

1 (a) Accumulative transport systems for glycine were identified in HeLa and Xenopus laevis kidney cells. In both cell types, influx and efflux obey Michaelis-Menten kinetics and kinetic constants were calculated for both parameters. A technique of 'lysing' and re-filling cells facilitated the measurement of efflux from intracellular concentrations of amino acid which were sufficiently high to cause carrier saturation. In agreement with most previous work, glycine is not involved in a system of exchange diffusion. The cells' ability to accumulate glycine is considered to be due primarily to the differences in apparent Km values for entry and exit.

(b) Replacement of extracellular Na ions with K, Li, or choline ions causes an increase in the apparent Km for influx and, in the absence of extracellular Na^+, cells are unable to accumulate glycine against its concentration gradient. Analysis of the Na^+-dependency of influx suggests that the rate-limiting step in influx involves the binding of a single Na ion per glycine molecule.

(c) In HeLa cells, an increase in intracellular Na^+ concentration causes a decrease in influx, mediated via a reduction in Vmax and, thus, carrier mobility. Influx in Xenopus cells appears to be insensitive to intracellular Na^+ levels but this may reflect a cell-type variation in the relative affinities of intracellular Na^+ and K^+ for substrate carrier site.

(d) Under the experimental conditions used, intracellular Na^+ concentration does not affect efflux of glycine from Xenopus cells. Efflux is, however, accelerated by increasing intracellular K^+. Results support the concept of energetic coupling between glycine transport and Na^+ and K^+ fluxes. The potential energy available from the alkali-metal electrochemical gradients is calculated to be sufficient to maintain the observed steady-state distribution ratios for glycine.

(e) No evidence was obtained for a direct link between cellular metabolism and glycine influx, in either cell type. Prolonged treatment with ouabain does, however, result in a diminished accumulative capacity. In HeLa cells this can be explained by the observed increase in intracellular Na^+ and a decreased Vmax of influx. In Xenopus cells alteration of intracellular Na^+ cannot account for the reduced accumulative capacity; so a direct effect of ouabain on efflux is not excluded.

2 The cells' ability to transport glycine was modified by varying their growth conditions.

If growth medium is replaced with fresh medium, glycine influx activity decays with a half-time of about 5 hours. The transport modification is associated with a reduction in Vmax and no change in the apparent Km of influx. This suggests that, although the structure of the carrier and its affinity for glycine remains unaltered, there is a reduction in either carrier mobility or the

number of functionally active carriers.

Regulatory control of a labile carrier could, therefore, be investigated.

3 Glycine transport was regulated by varying the serum concentration of growth medium. In both cell types, a progressive increase in the concentration of serum results in a proportional increase in influx and accumulation of glycine. T he alteration in influx is again associated with variation in Vmax rather than altered carrier structure. It is not known whether this effect is linked with the growth-regulatory action of serum but it does provide evidence for the existence of serum factor(s) concerned either directly or indirectly with amino acid transport activity.

Studies using cycloheximide support the existence of a labile carrier protein and indicate that protein synthesis is necessary for the serum-dependent increase in transport to occur.

4 A more rapid modification of transport was obtained by varying the concentration of substrate in the growth medium. An increase in the concentration of similarly transported amino acids during growth proportionally suppresses glycine influx, measured under fixed experimental conditions. The influx is minimal after growth in approximately normal growth-concentration of amino acid. Glycine influx is not influenced by the prior growth concentration of leucine.

Regulatory control is primarily via a change in Vmax of influx. Efflux is not significantly altered. Cycloheximide treatment again caused a decay in normal influx activity. Results are consistent with the view that membrane components directly involved in carrier mediated active transport are in a dynamic state of turnover and that the decay observed is a result of protein catabolism without replacement. Actinomycin D has no effect on normal influx within 7 hours, indicating a store of appropriate mRNA.

Both cycloheximide and AMD do, however, prevent the acquisition of additional transport activity observed during growth in reduced amino acid concentrations. Data indicate that protein synthesis is required for the initial regulatory signal and for derepression (or stimulation) of influx to occur. It is suggested that there exists a system of genetic regulation of carrier synthesis which is analogous to that for enzyme synthesis in bacteria. The mechanism by which the regulatory control receives its input was investigated. Intracellular amino acid pool size was manipulated either directly, by using the 'lysing and refilling' technique, or indirectly, by incubation in low-Na^+ medium. Both types of experiment indicated that pool size is not a major factor in the control mechanism. It is proposed that the limiting factor to control is the rate of membrane transport of substrate.

INTRODUCTION

Amino acid transport

Recognition of ion dependence

In 1952, Christensen and co-workers noted that the concentrative uptake of glycine and alanine into duck erythrocytes and Ehrlich Mouse Ascites tumour cells was strongly inhibited when external Na was replaced by K, (Christensen, Riggs & Ray, 1952; Christensen & Riggs, 1952), or choline, (Christensen, Riggs, Fischer, & Palatine, 1952). This, and the fact that amino acid uptake from a high external concentration resulted in an increase in intracellular Na and a decrease in intracellular K, indicated an interdependent relationship between non-electrolyte transport and ion movements. The hypothesis initially preferred was for an association between amino acid influx and K efflux. (Riggs, Walker, & Christensen, 1958). Amino acid accumulation then occurred only in the presence of adequate intracellular K levels. An alternative view was that part of the Na influx was via a ternary complex of carrier, amino acid, and Na ion; the Na electrochemical gradient providing energy for amino acid uptake. Amino acid uptake was reduced during metabolic inhibition but it was proposed that this was secondary to a disturbance in normal alkali metal distribution, (Riggs, Walker, & Christensen, 1958; Christensen, 1970).

Since these early observations, several models for non-electrolyte transport have been suggested, proposing an energetic coupling with K or Na fluxes or a combination of both. Controversy still exists as to whether electrochemical gradients of the alkali-metal ions provide the entire energy requirement, or whether there is direct coupling to cellular metabolism.

In 1960, Csáky presented evidence for a mucosal Na requirement for active sugar transport across the

isolated intestine of the toad, (Csáky & Thale, 1960). The qualitative similarity between Na-dependency in both amino acid and sugar transport systems suggested a common energy source and models concerning the role of Na appear to be equally applicable to either system.

Sugar/amino acid interaction

Glycine uptake into everted sac of rat intestine was found to be inhibited by non-metabolisable, actively-transported sugars, (Newey & Smyth, 1964). Neither non-actively transported sugars nor actively transported, but metabolisable, sugars (eg. glucose) inhibited amino acid uptake. This, metabolically linked, sugar/amino acid interaction indicated a common, limited cellular energy source, possibly ATP, (Hardcastle, Newey & Smyth, 1968). Alternatively: (1) the mutual competition for transport between different non-electrolytes has been explained in terms of a polyfunctional carrier, (Alvarado, 1968). Data showed that initial influx rates were affected, (ie before depletion of energy), and that counter-transport could be demonstrated between amino acids and actively transported sugars. On this basis, however, it is difficult to reconcile the inhibitory effect of phloridzin on sugar but not amino acid transport, (Segal, Blair & Rosenberg, 1963). In addition, not all workers have been able to find heterogeneous exchange, (Kimmich, 1973). For instance, accelerated efflux of hexose could not be induced by external glycine, a potent inhibitor of hexose accumulation in rat-kidney-cortex slices, (Genel, Rea & Segal, 1971). In rabbit ileum the stimulatory effects of sugar and amino acid on short circuit current (and thus, net mucosal to serosal flux of Na) were additive, indicating separate but parallel carrier mechanisms. (Schultz & Zalusky, 1965). (2) If the transport of substrate causes an increase in intra-cellular Na due to Na co-entry, it has been suggested that it is the resultant diminution in transmembrane Na-gradient which causes a reduction in the Na-

dependent transport of other non-electrolytes, (Frizzel & Schultz, 1971). In this case, only localised changes in Na-gradient may be necessary. Unfortunately, (in isolated chick cells), no correlation has been found between rate of transport, and hence the rate of dissipation of the Na-gradient, for one substrate, and the degree of inhibition of transport of a competing substrate, (Kimmich & Randles, 1973a).

Coupling of non-electrolyte and active Na transport

Direct coupling. Because Na is necessary for the active transport of at least 2 different substrate species, Csáky suggested that the Na requirement was not specific to each transport system, but general, in that it was required for Na-K-stimulated ATPase activity, (Csáky, 1963). All uphill transport processes then derived energy through the Na-K-pump and carriers were responsible for specificity within the transport system, (Csáky, 1961). The highly specific requirement for Na, rather than other monovalent or divalent ions, was similar for both non-electrolyte transport and Na-K-ATPase function, (Csáky & Thale, 1960; Csáky, 1961). Because of this, Schultz and Curran, (1970), suggested that the systems might have appeared simultaneously in the evolution of multicellular organisms.

Indirect coupling. More support has been given to indirect coupling between non-electrolyte transport and active Na transport. In the small intestine, passive Na movement across the mucosal membrane is followed by active extrusion at the serosal membrane. Csáky and Hara obtained evidence that the site of action of ouabain inhibition of mucosal-serosal transport of 3-O-methylglucose across the small intestine of bullfrog was at the serosal rather than the mucosal surface, (Csáky & Hara, 1965). The inhibitory action of ouabain was prevented by high serosal-K. This provided evidence that a functional Na pump was essential for sugar transport with ouabain acting at the site of active Na, rather than sugar,

transport. These workers, in support of their direct-coupling concept, concluded that, if Na were associated directly with non-electrolyte movement, then Na extrusion ought to be at the mucosal surface. If, however, it is assumed that mucosal extrusion of Na is not essential, then the physical separation of the loci for the ouabain-sensitive Na pump and the non-electrolyte pump, would invalidate a direct-coupling hypothesis. Similarly, ouabain was shown to affect the Na-sensitive mucosal-serosal flux of the amino acid alanine across rabbit ileum, when present at the serosal, but not the mucosal, surface, (Field, Schultz & Curran, 1967).

Levi has more recently obtained evidence for temporal as well as spatial separation of the 2 pumps, (Levi, 1972). The inhibitory effects of ouabain on γ-amino-butyric acid (GABA) or α-aminoisobutyric acid (AIB) uptake into chick brain tissue increased during development from embryo to chick in a manner which paralleled the increase in Na-K-ATPase activity. Uptake was inhibited by Na deficiency at all stages of development, so that the external Na effect appeared to be independent of ouabain-sensitive Na pump activity.

In addition, the finding that alanine influx across the mucosal border of rabbit ileum, although dependent upon extracellular Na, was independent of intracellular Na, does not appear to support Csaky's view; depletion of intracellular Na ought to inhibit active transport, (Schultz, Curran, Chez & Fuisz, 1967).

'Ion gradient hypothesis'

Crane, studying the initial uptake of sugars in hamster intestine, proposed the Na-gradient hypothesis, in an attempt to explain the findings that (1) entry kinetics could be described by the Michaelis-Menten equation, (2) accumulation was Na dependent, and (3) indirect coupling existed between active non-electrolyte transport and Na-K-ATPase activity, (Crane, Miller & Bihler, 1961). A ternary complex between

Na, sugar and carrier was proposed for the influx of actively transported sugars. The energetically maintained Na-gradient then provided energy for substrate accumulation via membrane carriers. The carrier was assumed to migrate across the cell membrane in either free or combined forms. Thus, in order to account for the transfer of energy from Na flux to substrate flux, allowing accumulation, rather than equilibration of substrate, Crane and co-workers suggested that the binding of Na to the carrier concomitantly increased the affinity of the carrier for non-electrolyte, (Crane, Forstner & Eichholz, 1965). Because of the asymmetric distribution of Na, it followed that carrier affinity for amino acid would be less at the internal surface, and net inward flux of substrate would occur until, (with increasing intracellular concentration), the degree of saturation of the carrier at the internal surface equalled that at the external surface.

In addition to decreasing the apparent Km of influx of non-electrolytes, evidence exists that the Na ion also causes an increase in Vmax, (a mobility plus affinity effect). The extent of the changes, (either Km or Km + Vmax), appears to vary with substrate and tissue, (Alvarado, 1972 ; Christensen, 1970; Lin & Johnstone, 1971). Heinz has pointed out that accumulation can occur only under very restricted conditions if carrier mobility, but not affinity, is altered; that is, when the product of the mobilities of carrier and ternary complex is greater than the product of the two possible binary complexes, and then only if the mobility of the empty carrier is greater than the mobility of the carrier/Na complex, (Heinz, 1972). These changes in carrier mobility have been offered as an explanation of the Na effect on Vmax of sugar influx in strips of rabbit ileum, (Goldner, Schultz & Curran, 1968).

The Na-gradient alone has not been shown to provide sufficient energy for non-electrolyte transport. It

is thought that a high internal K may act synergistically with a low intracellular Na, in a Na-K-gradient hypothesis. Bosackova and Crane suggested that K could compete with Na for the monovalent-ion binding-site on the carrier, rendering it less efficient, (Bosackova & Crane, 1965). Asymmetric K, as well as Na, distribution would then alter non-electrolyte accumulation as, normally, the carrier would tend to dissociate Na and bind K at the internal surface of the membrane, converting it to a form with low non-electrolyte affinity, (high Km). It was suggested that the K-carrier complex returned to the outer surface of the membrane, (Crane, Forstner & Eichholz, 1965).

Further evidence for an 'ion gradient hypothesis'

The ion-gradient hypothesis predicts:
(1) an associated movement of amino acid and sodium, and possibly potassium. Ion analyses of rat jejunal mucosa, (Brown & Parsons, 1962) and mucosal strips of rabbit ileum, (Koopman & Schultz, 1969), showed that, although intracellular Na did not vary significantly, K was depressed in the presence of up to 27mM of actively transported organic solutes, including alanine, 3-O-methylglucose, and galactose. In early work, Christensen noted that accumulation of amino acid into Ehrlich cells (from high external concentrations, eg 60mM), was accompanied by a loss in intracellular K and a gain in intracellular Na, (Christensen & Riggs, 1952). Later work from the same laboratories, using pigeon erythrocytes, presented evidence that intracellular levels of Na and K were not normally altered during amino acid uptake, (Wheeler & Christensen,1967). Neither influx nor efflux of K was affected, but both influx and efflux of Na were increased, so that no net movement of Na could be detected. In addition, those amino acids whose uptakes were most strongly stimulated by Na, caused the strongest stimulation of Na influx. The extra efflux activity was only slightly affected by

ouabain and, therefore, not associated with the normal extrusion of Na. It has been suggested that, if the additional Na efflux were associated with efflux of endogenous amino acids rather than influx of the experimental amino acid, this provided indirect evidence for the Na-gradient hypothesis, (Schultz & Curran, 1970). Schultz & Zalusky (1965), studying the short-circuit current across isolated rabbit ileum as an indication of active transport of Na, found an increase in transmural current if amino acid (alanine, glycine or methionine) were added to the mucosal surface. Ouabain prevented the increase in short-circuit current, if added at the serosal surface. The results indicated that Na was not merely acting as an activator for non-electrolyte transfer, but that Na movements occurred in association with amino acid uptake in the intestine. The Na/alanine interaction in rabbit ileum has since been extensively studied and evidence obtained for a model in which sequential binding of amino acid and Na to a transport site resulted in a ternary complex and co-transport of Na and alanine, without direct utilisation of metabolic energy. The model predicted that translocation of complex was reversible; that is, effluxes, in addition to influxes, of Na and alanine were linked, so that net alanine movements were influenced by the Na-gradient, (Curran, Schultz, Chez & Fuisz, 1967). Data showed that alanine influx was influenced by external Na, (Schultz, Curran, Chez & Fuisz, 1967), and efflux by internal Na, (Hajjar, Lamont & Curran, 1970). In addition, mucosal alanine caused an increase in Na influx, and the efflux of Na from cells to mucosal solution was affected by the presence of alanine inside the cell, (Curran, Hajjar & Glynn, 1970).

Vidaver, studying the uptake of glycine into 'lysed and restored' pigeon erythrocytes, again found that a direct link existed between amino acid uptake and Na influx, (Vidaver, 1964a). Ratios of between 1.5

and 2.2 were obtained when the increase in Na influx and Na-dependent glycine entry, due to a raised external glycine concentration, (from 0.3 to 3mM), were compared, (Vidaver, 1964c). This implied that 2 Na ions entered the cell in association with each glycine molecule, and agreed with the stoichiometric relationship obtained by kinetic analysis of the Na dependency of glycine uptake. The increment ratios could, however, be determined satisfactorily only if basal Na influx were restricted by reduction of external chloride, (Vidaver, 1964b).

(2) that the direction of net non-electrolyte movement be determined by the direction of the electro-chemical gradients of Na and K.

Supporting evidence was obtained when Vidaver, in the same studies described above, demonstrated that the magnitude and direction of net glycine movement in pigeon erythrocytes was determined by the magnitude and direction of the Na gradient. The latter was manipulated directly by modification of the lysing and restoring solutions. (Lysed and restored cells had a limited capacity for glycine accumulation, in that when external glycine was 0.6mM, a final distribution ratio of 2 was obtained, compared with 8 in intact cells. This could be explained by the cells' inability to actively maintain internal K and Na concentration. It was assumed that the transport mechanisms were qualitatively similar in both cell preparations, (Vidaver, 1964a).) External Na stimulated the uptake of glycine but had no direct effect on glycine efflux. Accumulation of amino acid was observed when a favourable transmembrane Na-gradient existed but not when intracellular Na exceeded the external concentration. The results appear to be limited in that only the initial gradients for Na and K were presented.

In rat diaphragm, a direct relationship was found between AIB influx and extracellular, but not intracellular, Na concentration, (Kipnis & Parrish, 1965).

The authors imply that this argues against the Na electrochemical gradient as a major determinant in active AIB transport; in fact, the hypothesis only infers that net movement of amino acid depends upon the gradient. Additional favourable evidence has been obtained during in vitro studies of intestinal transport. Net passive fluxes of Na determined the direction of amino acid movement, and amino acid (alanine) flux down its concentration gradient determined the direction of Na movement across mucosal strips of rabbit ileum, (Hajjar, Lamont & Curran, 1970; Curran, Hajjar & Glynn, 1970). Eddy, in studies of glycine movement in mouse ascites-tumour cells, has proposed that the potential energy for amino acid transport is inherent in the electrochemical gradients of both Na and K. His data showed that, after cyanide poisoning, Na-dependent glycine influx occurred and, although reduced, a net glycine accumulation which was dependent upon the Na-gradient, could be measured, (Eddy, Mulcahy & Thomson, 1967; Eddy, 1968a). Cellular Na was varied by varying external Na during preincubation with cyanide. An increased intracellular Na resulted in a decrease in the initial rate of uptake of glycine. Eddy and co-workers suggested that this could be due to either an increased efflux of radioactively labelled glycine; or a glycine influx governed by the rate of return of carrier to the outer surface of the membrane. External K reduced influx, probably competing with Na for carrier sites, (Eddy & Hogg, 1969). An effect of K-gradient was noted, in that when intra- and extra-cellular Na concentrations were equal, (30mM), the glycine gradient was larger if choline replaced external K, and an equation was proposed to explain the relationship:

$$\frac{(gly)_{in}}{(gly)_{out}} = \frac{(Na)_{out}}{(Na)_{in}} \cdot \frac{(1 + \theta(K)_{in})}{(1 + \theta(K)_{out})}$$

(Eddy, 1968b). θ was less than 0.025, ie, the effect of K was small compared with that of Na.

Evidence against a simple ion-gradient hypothesis

Some of the data obtained were inconsistent with the ion-gradient hypothesis. Controversy has arisen as to the relative importance of the alkali-metal ion gradients and cellular ATP as sources of energy for non-electrolyte transport.

Mouse ascites-tumour cells, when treated with NaCN but with normal ion gradients maintained, accumulated glycine to only about 20% of the normal distribution ratio obtained during respiration, (Eddy, 1968). This was indicative of a limiting-factor other than the Na plus K gradients. When the equation which Eddy proposed to explain glycine accumulation in terms of both Na and K gradients, was applied to methionine transport in ascites cells, then values of θ were obtained of 0.047 during respiration, and 0.01 in the presence of metabolic inhibitors, (Reid & Eddy, 1971). This suggested that the coupling of the K-gradient to methionine transport had a metabolic requirement.

A direct link between cellular metabolism and glycine transport in Ehrlich cells has been further investigated, (Potashner & Johnstone, 1970; 1971). Cells were preincubated with varying Na and K concentrations, with and without DNP, in order to obtain varying intracellular alkali-metal ion and ATP contents. In the absence of ATP, the cells were able to accumulate glycine against its concentration gradient in a manner which was dependent upon Na, or Na + K, gradients. In the presence of ATP, however, (with normal extracellular Na) accumulation was very much increased and elevation of intracellular Na had little effect on the steady state value. This indicated that cellular ATP was a more important energy source for amino acid accumulation than the ion gradients. Both extracellular Na and cellular ATP decreased the apparent Km of influx without altering Vmax, which suggested an effect via activation of the substrate carrier, (Potashner & Johnstone, 1971). Later work, on the

same cell type, determined the energy contribution of the K-gradient, (Johnstone, 1972). Both Na and K gradients were manipulated and the available potential energy calculated as $2.3RT\log(\frac{Na_o}{Na_i} \cdot \frac{K_i}{K_o})$ cal/mole. This was compared with the theoretical energy required for observed glycine distribution ratios, using an external glycine concentration of 0.1mM, ie., $2.3RT\log(\frac{glycine_i}{glycine_o})$. The maximum available energy was about equivalent to that required for glycine accumulation but, when external K was increased, a deficit increased, due to a reduction in gradient energy, with no accompanying effect on the steady state level of glycine. It appeared, therefore, that the K gradient was not a major source of energy for glycine accumulation. Influx was, however, reduced by a high external K. This could be explained if K-binding to carrier site reduced the affinity of the carrier for amino acid; as previously predicted by the ion-gradient hypothesis. Johnstone suggested that, although the affinity for carrier site for K was less than that for Na at the external surface, the relative affinities could be reversed at the internal surface; thus K remained bound at the internal surface despite high intracellular Na. This explained the lack of effect of intracellular Na. A possible role for ATP would be to maintain the asymmetric affinities, enhancing the competitive effect due to the asymmetric ion. distribution.

Again in Ehrlich cells, the energy expenditure needed to maintain steady state AIB concentration gradients has been calculated and compared with the energy available in the alkali-metal gradients, (Jacquez & Schafer, 1969). Assuming a 1:1 relationship, the energy available in the Na electrochemical gradient alone did not satisfy the requirement. Results were, however, consistent with the formation of a K/carrier complex at the inner surface of the membrane

and outward movement of the complex, so that the return of the carrier to the outer surface of the membrane could be driven by the K electrochemical gradient. A model based on both Na and K gradients satisfied the energy requirement but 100% efficiency of coupling between ion fluxes and AIB movements was needed. This, and the fact that some accumulation occurred despite reversal of both Na and K gradients, suggested an additional source of energy. The possibility that AIB accumulation, in excess of ion gradient potential, was via exchange with endogeneous amino acid was excluded in later work, after measuring the amino acid pool, (Schafer & Heinz, 1971). These workers presented evidence that the transient gradient of AIB into EAT cells (at AIB steady state) increased as the electrochemical gradients of Na and K increased, showing some agreement with the ion-gradient hypothesis. A better correlation was obtained using the sum of the Na and K electrochemical potential gradients rather than that of Na alone. AIB was, however, transported against its concentration gradient, despite reversal of the monovalent ion gradients. Again, this suggested an additional driving force. Schafer and Heinz calculated that the flux of AIB could be reversed only if the opposing energy were of the order of 4000 joules.mole^{-1} and suggested that AIB transport was directly coupled to metabolism, giving an additional driving energy at least 4000 joules.mole^{-1}. One possible source of error discussed was that the measured intracellular concentration of Na was greater than the cytoplasmic (free) concentration and that the effective transmembrane gradient was, therefore, greater than that calculated.

One of the arguments used in favour of the ion-gradient hypothesis is that ouabain does not affect the initial rate of transport of non-electrolyte but, due to blocking of Na-K-ATPase, affects the accumulative capacity of the cell. This would be mediated via a

time-dependent reduction in cellular ion gradients, and, hence, the asymmetry of the carrier for substrate, (Schultz & Curran, 1970; Charalampous, 1971). In mouse pancreas, however, ouabain (10^{-4}M) immediately decreased glycine influx by 40%, without a substantial effect on Na and K gradients, (Lin & Johnstone, 1971). This tissue, in vitro, was unable to maintain a transmembrane Na-gradient and yet glycine could be accumulated 10-12X from an external concentration of 2mM. Variation in extracellular Na and K showed that although influx was directly proportional to extracellular Na concentration, the K gradient, like the Na gradient, was unlikely to be a major determinant of net glycine transport. Comparing the transmembrane potential energy available from the gradients with that required to maintain glycine steady state values, Lin & Johnstone, assuming a 1:1:1 relationship, calculated that the energy requirement could be 2-3X that available. DNP treatment resulted in reduction in cellular ATP and an associated inhibition of glycine influx. The inhibition could be partially overcome by addition of glutamine or glutamate; this was explained by the ability of the corresponding keto acid to support substrate-level phosphorylation. Data, therefore, suggested that cellular ATP could support amino acid transport and that ouabain had a direct inhibitory action.

Ouabain has also been shown to have a direct effect on galactose influx and efflux across brush-border of rabbit ileum, (Holman & Naftalin, 1974). Replacement of external choline with Na resulted in an increased influx which was partially prevented by ouabain. An increase in extracellular Na also led to an increased intracellular Na. This, in terms of the ion-gradient hypothesis, ought to have caused an increased efflux of galactose. In fact, a decrease was observed and this decrease was prevented by ouabain.

Baker and Potashner have shown that the Na-dependent influx of glutamate into squid axon and crab walking-

leg nerve was reduced by cyanide and DNP, despite the existence of appreciable alkali-metal ion gradients, (Baker & Potashner, 1973a,b). Efflux of glutamate from squid axon was shown to be also dependent upon cellular metabolism, in that cyanide had an inhibitory effect which was relieved by injection of ATP. There appeared, however, to be sufficient energy available in the Na gradient alone to maintain the intracellular levels of glutamate in these tissues, (Baker & Potashner, 1973b). In crab walking-leg nerve, ATP depletion increased the apparent Km of influx, with no change in Vmax. These latter two findings indicated that the ATP requirement for influx and accumulation of amino acid was not obligatory but ATP had a direct effect on the transport system by increasing its efficiency.

An alternative to the ion-gradient hypothesis

In isolated chick-intestinal epithelial cells Kimmich demonstrated that galactose (Kimmich, 1970), and 3-O-methylglucose (Kimmich & Randles, 1973a), transport involved a direct input of metabolic energy. The sugars could be actively transported despite reversal of the Na and K gradients and 0.5mM ouabain partially blocked the influx within 30seconds. Similarly, valine was shown to be accumulated against its concentration gradient during a condition of outwardly-directed Na-gradient, (Tucker & Kimmich, 1973). Kimmich suggested that the energisation of non-electrolyte transport by the alkali-metal ion gradients was of limited importance when compared with a direct energy-input. He accounted for the characteristics of the transport systems in an alternative hypothesis in which the energy for various transport systems (amino acid, sugar and monovalent ion) was associated with a membrane-bound phosphorylated intermediate derived from ATP, (Kimmich & Randles, 1973a; Kimmich, 1973). The system was dependent upon Na, in that the latter was required for ATP hydrolysis and possibly, for altered carrier mobility, or affinity for substrate. K became

inhibitory to non-electrolyte transport as, in the presence of K, energy was utilised for monovalent ion transport. The ouabain block was at the level of the phosphorylated intermediate. Kimmich has also used his model to explain the sugar/amino acid transport interaction (discussed earlier) in terms of a common, limited, energised intermediate rather than a common carrier, (Kimmich & Randles, 1973b). Kimmich's model cannot, however, satisfy all the observed data for transport. For instance, the envisaged direct coupling between monovalent ion transport and non-electrolyte transport does not correlate with the observed spatial separation of transport loci in the intestinal cell. Also, the finding that an increase in active Na-transport accompanied non-electrolyte transport argues against this proposed model. According to the latter, active accumulation of non-electrolyte should decrease active Na extrusion; or, if the energy supply were not depleted, at least have no effect. Recently, confirming earlier findings, ouabain-sensitive Na efflux has been shown to be stimulated by the actively transported sugars, glucose, galactose, and 3-O-methyl glucose, in rat isolated intestinal epithelial cells, (Gall, Butler, Tepperman & Hamilton, 1974). The increased efflux could not be explained by an increase in available metabolic energy as 3-OMG is non-metabolisable.

Summary

The energy available in the electrochemical potential gradients of the alkali-metal ions has been calculated to be just sufficient to account for that required to maintain non-electrolyte steady-state distribution levels but this suggested an extremely high coupling efficiency. The coupling efficiency between amino acid transport and associated Na influx (rather than total Na influx) in Ehrlich cells has, however, been calculated to be adequate, (Heinz & Geck, 1974).

Evidence indicated that, in addition to that available from the ion electrochemical gradients, energy was

directly available from cellular metabolism for non-electrolyte transport. Alternatively, cellular ATP acted to enhance the efficiency of the coupling between non-electrolyte and alkali-metal fluxes.

Recently, however, the linkage between amino acid transport and ATP hydrolysis has been studied directly (in Ehrlich cells) by observing ATP hydrolysis in the presence or absence of AIB, (Geck, Heinz & Pfeiffer, 1974). In this instance, accumulation of amino acid did not appear to be directly energised by ATP metabolism, which inferred an unidentified additional energy source to account for the cells' ability to accumulate amino acids despite reversed alkali-metal ion gradients.

Exchange Diffusion

The movement of amino acid across the cell membrane can occur via exchange diffusion as well as by active transport and simple diffusion. That is, an amino acid on one side of the membrane can stimulate movement of an amino acid initially located on the other side of the membrane. One hypothesis is that the loaded and unloaded carriers have different mobilities, the loaded carrier moving much faster so that the uni-directional forward flux is stimulated by the presence of amino acid on the opposite side of the membrane, (Heinz, 1967). This 'counterflow' effect was first observed in the influx of glycine into Ehrlich ascites tumour cells, (Heinz, 1954). 1min influx from an external concentration of 2.5mM was doubled when the cells were preincubated for 10min with 10mM glycine.

Later work, studying the interactions amongst neutral amino acids during their uptake into Ehrlich cells, showed varying affinities and competition for transport, (Oxender & Christensen, 1963; Christensen & Laing, 1965). At least 6 categories were determined, (Christensen, 1968). The 2 principal were classified

as 'A', alanine-preferring (favouring the transport of alanine, glycine, serine, threonine, proline, asparagine, glutamine and methionine), and 'L', leucine-preferring (favouring the transport of leucine, isoleucine, valine, phenylalanine and methionine). 'A' mediated amino acids had a final distribution ratio of about 20, with an uptake which was relatively slow compared with 'L' mediated. In the latter group, however, accumulation was only about 2x, so that different modes of efflux were suggested. Efflux into an amino acid-free solution was similar for glycine and leucine but the efflux of leucine could be accelerated by increasing the external concentration of leucine. Thus the systems were further categorised as 'A' mediated, resembling active transport, and 'L' mediated, resembling exchange diffusion. There was extensive overlap; all amino acids, except possibly glycine, using both mechanisms, (Christensen & Laing, 1965). Similar conclusions have been reached in Novikoff hepatoma ascites tumour cells, (Belkhode & Scholefield, 1969). Transport was minimised by incubation at low temperatures, so that the contribution of exchange diffusion to total transport could be more easily studied. At 20°C, external methionine and ethionine (5mM) stimulated the efflux of radioactively labelled ACPC, independent of the presence of Na or K in the incubation medium. AIB and proline had no effect on the methionine stimulation of ACPC and were unable to themselves give rise to any significant exchange diffusion. At 37°C, methionine, AIB and proline competed during transport, indicating that methionine entered the cell via both 'A' and 'L' sites and thus supporting the evidence for either several carriers or several sites on one carrier.

Exchange diffusion of methionine, (Johnstone & Scholefield, 1965), and tryptophan, (Jacquez and Sherman, 1965) in EAT cells is not affected by metabolic inhibitors, further distinguishing it from

active transport. This was cited by Johnstone as evidence that the Na requirement for uptake is associated with the energy-dependent net transport rather than simple translocation of amino acid. In the latter case, it was suggested by Jacquez that, if the metabolic inhibitors were to act only at the free carrier, the results could be explained by considering exchange diffusion as displacement of one amino acid for another without a free carrier intermediate. Unlike Christensen, Jacquez interprets his results in terms of a single carrier (with several binding sites), (Jacquez, 1961).

Exchange diffusion has also been demonstrated for L-phenylalanine in cultured hamster cells, (Hare, 1967), for methionine in mouse pancreatic slices (Clayman & Scholefield, 1969) and for histidine in S37 Ascites tumour cells, (Matthews, Leslie & Scholefield, 1970).

Evidence, therefore, exists that an amino acid is able to react with 2 or more transport sites. With the exception of Heinz, most workers have found that glycine was not involved in exchange diffusion, (Piperno & Oxender, 1968; Schafer & Heinz, 1971).

Serum effects

The growth of cells in culture is affected by genetic properties, cell density and also by the stimulatory activity of serum, (Clarke & Stoker, 1971). Growth ceases (the cells remaining in G1 phase of the cell cycle) at a saturation density which is characteristic of the cell type and the concentration of serum in the culture medium, (Clarke & Stoker, 1971; Jainchill & Todaro, 1970). Addition of serum to a saturation density culture initiates DNA synthesis, and cell division of a limited number of cells, after a variable time lag, (Todaro, Lazar & Green, 1965). Growth-promoting activity has been shown in all sera tested and has little species specificity, (Clarke & Stoker,

1971). The factor initiating growth is non-dialysable (Todaro et al., 1965) and can be precipitated with the gamma-globulin fraction (Jainchill et al., 1970) although it is not, itself, a gamma-globulin. Serum activity is depleted during cell proliferation but studies are complicated by additional factors produced by the cells themselves, (Clarke & Stoker, 1971).

It was possible that density-dependent inhibition of growth was correlated with changes in membrane permeability and transport. The membrane would thus be intimately involved in growth regulation. Hare studied the transport of phenylalanine (an L-mediated amino acid) in normal and polyoma transformed hamster cells, (Hare, 1967). Although the latter cell type showed diminished contact inhibition, he found no significant differences in transport properties between the two cell types. In contrast, Foster found that accumulation of the non-metabolisable amino acid AIB was greater in PY3T3 cells (not sensitive to 'density-dependent' inhibition of growth) than in 3T3 cells (showing contact inhibition), (Foster & Pardee, 1969). Confluent 3T3 cells accumulated AIB at a lower rate than non-confluent but when high density cultures were maintained in a confluent state by growth in 50% serum, compared with the normal 10%, then transport was equal. It was thus apparent that AIB accumulation was reduced only when growth had stopped but it was not established whether the finding was the cause of, or a consequence of, cessation of growth. The result was limited to non-metabolisable amino acids.

As serum is involved in growth regulation, associated effects of sera on membrane transport have been reported. Phosphate and uridine (RNA precursors) transport into confluent 3T3 cells was stimulated by adding serum in fresh, or 3T3-altered, medium, (Pariser & Cunningham, 1971). In the latter case stimulation was less and it was suggested that confluent cells release into the medium a dialysable

inhibitor of transport whose effect is counteracted by serum. Serum had an additional stimulatory activity which was depleted equally by confluent and subconfluent cultures.

Most work has been to study serum repletion of cells grown in serum-free conditions. (^{3}H) uridine uptake into mouse embryo cells, (Hare, 1972a and b) and both normal and polyoma-transformed hamster embryo cells (Lemkin & Hare, 1973) was stimulated by addition of serum to cells previously grown in serum-free conditions. This was characterised by an increased Vmax with no change in the apparent Km and the continual presence of serum was necessary to maintain this increased activity. A greater turnover of phospholipid was recorded in hepatoma-tissue-culture cells when calf serum was added after 14-16hr depletion, (Knox & Pasternak, 1973).

The serum stimulatory effect does not seem to involve all transport systems; for instance, phenylalanine transport into 'serum-less' mouse embryo cells was not affected 6hr after the addition of serum, (Hare, 1972a).

In view of these results and existing evidence for serum factors, it seemed of interest to investigate the direct effects of growth in varying concentrations of serum on the glycine transport systems characterised for HeLa and Xenopus laevis kidney cells. Previous work in the laboratory had shown that some factor in normal calf serum caused an increase in the number of sodium pumps in HeLa cells, within 8hr, (Lamb, Boardman, Newton & Aiton, 1973).

Growth in reduced amino acid concentrations

This work was begun in an attempt to determine whether serum stimulation of transport was a consequence of higher extracellular amino acid concentration during growth.

In 1972 Gazzola reported that, in both isolated cardiac cell suspensions and intact preparations from chick embryo heart, the A-mediated amino acid transport activity (represented experimentally by AIB uptake) increased with the time of incubation in amino acid-free Krebs-Ringer bicarbonate buffer, (Gazzola, Franchi, Saibene, Ronchi & Guidotti, 1972). This increase was apparent within 1hr and was prevented if A-mediated, but not L-mediated, amino acids were present during the incubation. The work provided evidence that the A-mediated amino acid transport system was regulated in some manner by its substrates. Kinetic analysis of uptake showed that control was via an altered Vmax rather than Km. Inhibitors of RNA translation (cycloheximide) and RNA transcription (AMD) prevented the increase and caused a progressive decrease in transport activity.

Later studies from the same laboratories described the relative needs for mRNA transcription and translation in the regulatory mechanism, (Franchi-Gazzola, Gazzola, Ronchi, Saibene & Guidotti, 1973). Cells were pre-incubated in amino acid-free conditions in the presence of cycloheximide; that is, with the stimulus for new transport activity but in a condition of inhibited mRNA translation. Subsequent removal of cycloheximide resulted in an increased AIB uptake, in the absence of amino acids. Although reduced, the increase persisted in the presence of the A-mediated amino acid alanine or AMD, added separately but not together. It appeared that transcription of mRNA, for one or more proteins involved in A-mediated amino acid transport, occurred during cycloheximide treatment without amino acids and that protein synthesis occurred during the subsequent incubation without cycloheximide. A-mediated amino acids, present during the preincubation, prevented this, suggesting that substrate control was at the level of specific mRNA transcription. Alternatively, in the presence of amino acid, both mRNA and a

repressor mRNA were produced and the latter activated during the transl__ation phase. Repressor would be inactivated, or not produced, in the absence of A-mediated amino acids.

The regulatory control was partially independent of external Na in that when choline replaced Na during preincubation in amino acid-free medium the time-dependent increase was reduced (from 4 to 2X the base level) but not abolished, (Gazzola, Franchi-Gazzola, Ronchi & Guidotti, 1973). Of the 4 systems for transport of neutral and basic amino acids tested, 3, namely Systems ASC, L and Ly^+, did not exhibit adaptive control.

Several authors have noted a similar increased amino acid transport activity after preincubating tissue but none have associated the effect with low amino acid concentrations - that is, with a lack of transportable substrate; for instance, AIB uptake increased when immature rat uterus was incubated in Krebs-Ringer bicarbonate buffer, (Riggs, Pan & Feng, 1968). Similar increases in transport activity were found for L-proline and L-alanine but not for L-valine, L-phenylalanine or L-leucine, (L-mediated amino acids). The increased uptake was characterised by an increased Vmax with no change in Km and was prevented if Na was replaced by choline during the preincubation, or if puromycin was present, (Riggs & Pan, 1972).

In human placental tissue AIB uptake increased to give, after 3-5hr preincubation, distribution ratios 25X external level, compared with 5X in non-incubated tissue, (Smith, Adcock, Teasdale, Meschia & Battaglia, 1973). Tews has suggested that the increase in AIB uptake she and co-workers found in rat liver slices, after preincubation in Krebs-Ringer, may have reflected an increase in effective endogenous cyclic AMP levels, (Tews, Woodcock & Harper, 1970). The latter workers, in contrast to others, found that only cycloheximide, and not AMD, prevented the increased

uptake and suggested that at least part of the protein synthesis necessary did not require newly formed mRNA.

Richelson has reported that if mouse neuroblastoma cells were starved for 48hr of a particular amino acid, then the transport activity for that amino acid was increased (a reduced apparent Km was recorded), (Richelson & Thompson, 1973).

In the light of the findings of Gazzola et al., Christensen has reviewed his earlier work on guinea pig liver, (Christensen, 1973). He had found that the accumulative ability for glycine and AIB increased to give a 4-fold increase in distribution ratio, within 24hr after birth. Although no direct evidence was given, Christensen has suggested that the stimulating (or derepressing) factor for transport development may be the fall in plasma amino acid levels which accompanied birth.

In 1962, after an extensive study of the free amino acid contents of tissues, Roberts found that characteristic distributions of amino acids were retained even when major disturbances were induced in the homeostatic mechanisms of the animal as a whole, (Roberts & Simonsen, 1962). He concluded that 'remarkable biological servo-mechanisms' must exist to regulate a continuous adjustment of distribution between intra- and extra-cellular compartments.

This cited evidence, and that to be presented, shows that such a control of regulation of the active transport of amino acids does exist.

Tissue Culture

History

Tissue culture can be described as the growth of tissue fragments in isolation. There is a loss of tissue organisation and peripheral growth of cells which can then be observed. Cell culture is classified under the

heading of tissue culture and results from total loss of organisation of tissue and cell dedifferentiation to give a uniformly similar population.

Although tissue culture was first recorded in 1866 when von Recklinghausen succeeded in culturing amphibian blood cells for up to 35 days, there is a possibility that he and contemporary workers were merely delaying cell death, (Paul, 1970). In 1907 tissue culture was recognised and accepted when Ross G. Harrison, using the 'hanging-drop' technique, was able to culture nerve tissue from the spinal cord of tadpole (in clotted lymph from frog) and subsequently observe the growth of axons into the medium, (Willmer, 1966).

When Alexis Carrel introduced aseptic techniques into tissue culture in 1913 the field became open to many more workers. It is reported that Carrel was able to keep connective tissue cells from chick embryo heart in active division for 34 years (Paul, 1970); but evidence now suggests that chick fibroblasts will not survive in culture and that these early cultures were probably seeded with living cells as the old cultures were fed crude extract from chick embryos, (Hayflick, 1968).

By 1914 tissue culture techniques had been used to study cytological structure, cell movement and cell division and had provided evidence for the neurone theory of the nervous system and the myogenic theory of heart beat. Growth requirements were investigated and defined media developed. An entirely synthetic medium has never been realised and, with the exception of one or two cell types, cells need a biological extract (eg serum) for prolonged culture. In the 1920's research fell into two main streams, (a) embryonic tissue development - notably in the laboratories of Strangeways and Fell - and (b) cell growth, multiplication and nutrition.

Cell culture has progressed with advances in other fields, eg:

Phase contrast microscopy in the study of chromosomal activity, cell surface and pinocytosis.

Electron microscopy, eg: the finding that collagen fibres are found near the surface of fibroblasts.

Chromatography and microanalysis in studies of cell metabolism.

In addition, tissue culture has led to interest and the possibility of research in other fields, eg:

Cell differentiation during embryonic development.

Cancer research by viral induction of cancer in controlled 'in vitro' conditions.

Chromosomal variation in relation to human abnormalities.

Studies of aging in euploid cells.

The investigation of protein synthesis inhibitors.

Membrane transport studies by radioactive tracer techniques.

General information

Cells for culture are obtained (a) from tissue cultured on a plasma clot medium, (cells grow peripherally into capillary tubing, which is then broken, and cell colonies separated) (b) by mechanical or enzymatic disruption of tissues to give cells in suspension, (cell types are separated by differential centrifugation). Cells are grown either as suspensions in fluid in rotating vessels or directly on glass (or plastic) as a monolayer, with a fluid medium. When the culture is innoculated into new culture vessels a primary cell line is obtained. Some lines develop the potential to be sub-cultured indefinitely and are then classified as established cell lines. The transition from primary to established may be gradual or may occur by transformation. Some primary cell

lines, eg. human fibroblasts, never undergo spontaneous transformation but have a defined number of divisions before death, (Hayflick, 1968). Mouse fibroblasts, however, will always undergo spontaneous transformation about 3 months after the primary line is established.

Established cell lines generally have similar characteristics in that they have a doubling time of 12-24 hours, grow to high densities, have little spatial orientation and can be established in suspension cultures. There is usually loss of specialised functions, eg. kidney epithelial cells lose their brush border and there is a loss of hormone production from endocrine cells. There are some exceptions, eg. fibroblasts generally retain their capacity to secrete collagen and antigens controlling species-specificity are retained.

The HeLa strain was established as an epidermal cell line by Gey in 1952 (Gey, Coffman & Kubicek, 1952) from human cervical carcinoma tissue. The cells are typical of cancer cells in that they have abnormal chromosome numbers, between 50 and 350 chromosomes per cell, compared with the normal 46, (Hayflick, 1968). The cells tend to be hypotetraploid. Aneuploidy (not a simple multiple of the haploid number of chromosomes) occurs in HeLa cells mostly as a result of a multipolar spindle at metaphase of mitosis. The frequency of multipolar spindles is about 35% of the dividing cells during the first few days of subculture, tripolar being the most common type, (Hsu & Moorhead, 1956).

Xenopus laevis kidney cells (line A-6) were established in 1965 by Rafferty and are adult epitheloid cells, (Rafferty, 1969). The line exhibits aneuploidy and is described as 'vigorous' compared with similar amphibian lines which remain euploid. The modal chromosome number is 45, compared with the diploid number of 36. The minimum doubling time is 22 hours. It is thought that aneuploidy and abnormal gene combinations may be beneficial to cell survival in culture in an artificial environment.

MATERIALS

HeLa cells (clone S-3) were obtained from Flow Laboratories Ltd., and kept as a frozen stock in a Union Carbide LR-40 Liquid Nitrogen refrigerator or subcultured in the laboratory.

Xenopus cells (line A-6) were originally obtained from the Zoology Department of this University.

B.M.E. growth media, glutamine, calf and foetal bovine serum, balanced salt solutions and amino acid and vitamin concentrates were from Flow Laboratories Ltd. W&Q growth media and trypsin were from Biocult Laboratories Ltd. Gentamicin was from Roussel Laboratories Ltd.

Glucose was purchased from Boots Co.Ltd. and sorbitol from Laporte Industries Ltd.

Tris base, cycloheximide, actinomycin D, adenosine triphosphate (di-sodium salt), L-leucine, α-aminoisobutyric acid, and D-cysteine hydrochloride were bought from Sigma Chemical Co. Ouabain glucoside was from the Laboratoire Nativelle, Paris. The liquid scintillator was NE 250 from Nuclear Enterprises Ltd. (2-^{3}H) glycine was obtained from the Radiochemical Centre, Amersham. The remaining chemicals and drugs, including 2,4 dinitrophenol, iodoacetic acid and iodoacetamide, were purchased from British Drug Houses.

Table 1

Basal Medium Eagle (BME)

Amino acids	mg/l	Vitamins	mg/l
L-arginine HCl	21.1	D-biotin	1.0
L-cystine	12.0	D-Ca-pantothenate	1.0
L-glutamine	292.0	choline chloride	1.0
L-histidine HCl	9.6	folic acid	1.0
L-isoleucine	26.2	i-inositol	2.0
L-leucine	26.2	nicotinamide	1.0
L-lysine HCl	36.5	pyridoxal HCl	1.0
L-methionine	7.5	riboflavin	0.1
L-phenylalanine	16.5	thiamine HCl	1.0
L-threonine	23.8		
L-tryptophan	4.0		
L-tyrosine	18.1		
L-valine	23.4		

Inorganic salts + other components (Earle's salts) mg/l

NaCl	6800
KCl	400
$MgSO_4 \cdot 7H_2O$	200
$CaCl_2 \cdot 2H_2O$	264
$NaH_2PO_4 \cdot H_2O$	140
$NaHCO_3$	1680
glucose	1000
phenol red	17

Table 2

Wolf & Quimby's amphibian culture medium (W&Q)

Amino acids	mg/l	Vitamins	mg/l
L-arginine HCl	69.50	choline chloride	0.550
L-cystine	13.20	folic acid	0.550
L-glutamine	161.00	i-inositol	1.100
L-histidine HCl.H_2O	23.10	nicotinamide	0.550
L-isoleucine	28.60	D-Ca pantothenate	0.550
L-leucine	28.60	pyridoxal HCl	0.550
L-lysine HCl	40.20	riboflavin	0.055
L-methionine	8.32	thiamine HCl	0.550
L-phenylalanine	18.20		
L-threonine	26.20		
L-tryptophan	5.62		
L-tyrosine	19.80		
L-valine	25.80		

Inorganic salts and other components mg/l

NaCl	3740	glucose	800
KCl	320	phenol red	8
$NaH_2PO_4H_2O$	100	$NaHCO_3$	1760
$MgSO_4.7H_2O$	160	Whole egg ultrafiltrate (mls)	100
$CaCl_2$ (anhyd.)	160		

Table 3

Versene solution	mg/l
Na EDTA	200
NaCl	6000
KCl	200
Na_2HPO_4	1065
phenol red (0.5%) (filter sterilized)	1 ml

Table 4

Components	HeLa Krebs	HeLa Ca-sorbitol	Xenopus W&Q salts	Xenopus Ca-sorbitol
NaCl	137 mM	-	64 mM	-
KCl	5.4 mM	-	4.3 mM	-
$CaCl_2$	2.8 mM	2.8 mM	1.4 mM	1.4 mM
$MgSO_4 \cdot 7H_2O$	1.2 mM	1.2 mM	0.7 mM	0.7 mM
NaH_2PO_4	0.3 mM	-	0.6 mM	-
KH_2PO_4	0.3 mM	-	-	-
HCl (M)	12 ml	12 ml	12 ml	12 ml
Tris base	1660 mg/l	1660 mg/l	1660 mg/l	1660 mg/l
glucose	1000 mg/l	-	1000 mg/l	-
sorbitol	-	49950 mg/l	-	35000 mg/l
calf serum	10 ml	-	-	-
foetal bovine serum	-	-	10 ml	-

Composition of experimental salt solutions.

METHODS

Subculture of cells

HeLa

All culture methods were carried out, aseptically, in a 'Bassaire' laminar flow cabinet. Routine growth medium was Basal Medium Eagle (B.M.E.) (see Table 1) supplemented with 10% calf serum. Gentamicin base was added to give a concentration of 0.04mg/ml. The cell line was maintained at 37°C as roller cultures, or in Roux bottles, seeded at 50,000 cells/ml medium. For experimental purposes, the cells were grown as monolayers on plastic Petri dishes (plates) - diameter 9cm.

'Plating-out'. Medium was drained from a Roux bottle and 5ml trypsin (0.025% in Mg/Ca-free Earle's balanced salts solution) added to the cell layer. The roux was incubated at 37°C until the cells began to separate. The trypsin was then neutralised by adding a small, known, volume of medium and the cell clusters broken, by blasting with a syringe, to give individual cells. Medium was added to the cell suspension to give a final concentration of 50,000 cells/ml and 10ml aliquots delivered to plates using a 'Brewer' automatic pipetting machine (BBL model 40). The plates were placed in plastic boxes, equilibrated with a 95% air/ 5% CO_2 mixture and sealed with autoclave tape. Cells were grown to monolayers by incubating for 3 to 4 days at 37°C.

Xenopus

Similar techniques were employed for Xenopus cells, with some modifications. The growth medium was Wolf & Quimby's amphibian culture medium (W&Q) (see Table 2), normally supplemented with 10% foetal bovine serum. Growth temperature was 26°C. During subculture, the cell layer was rinsed with Versene solution (for components, see Table 3) to bind free Ca, prior to

adding the dilute trypsin. The cells were grown in an air atmosphere.

Routine measurements

Cell number

Plates were washed, drained and the cell monolayer dispersed by adding 1ml trypsin (0.25% in Mg/Ca-free Earle's balanced salts solution) and incubating for approximately 5 minutes. The trypsin was neutralised by adding 4ml Krebs, or W&Q salt solution, and any cell clusters remaining were separated by blasting with a syringe. Samples of cell suspension were diluted with ISOTON and counted on a Coulter counter, model Z_F, or by using a Coulter Channelyser. Allowing for the dilution factor, the number of cells was taken as the integration of the curve produced from particle number against particle size. Accurate 'plating' of cells and a constant plating efficiency resulted in a uniform number of cells per plate, (in a randomly chosen experiment, the S.E. was 1% of the mean). It was thus possible to use a mean value in calculations when the cell number could not be estimated for each individual plate; eg: in measuring ion contents, when the extracellular extraction medium was Na/K-free.

Cell volume

Cell suspensions were obtained by trypsinisation, as previously described.

(1) Cell diameter was measured using a calibrated micrometer eyepiece. Assuming that trypsinised cells have a volume comparable to that of normal (flattened) cells but are spherical (Burrows & Lamb, 1962) cell volume was calculated from $4/3\pi r^3$, (where r = radius). Generally, the volume of 20-40 cells was measured and the mean volume used in calculations. This value agreed with that obtained by method (2).

(2) Total cell volume per plate was measured routinely (with cell number) using a Coulter counter, model Z_F,

with a Channelyser C1000, (Reed, Hughes, Taylor & Bruce, 1969). The mean cell volume could then be calculated. The instrument was calibrated using latex spheres of known volume. Cell suspensions were diluted with ISOTON and the volume measured within 20min. In a single count, a curve of cell number against cell size (or channel number) could be obtained and the curve integrated, using Simpson's rule, (Boardman, Huett, Lamb, Newton & Polson, 1974).

Intracellular water estimation

In several experiments the volume of normal cells was compared with that of cells placed in a medium of defined increased osmotic strength. Assuming that the dry weight remained constant and that, in hypertonic solutions, the cell behaved as an osmometer, cell water was calculated from $\frac{V_1 - V_2}{(1 - \pi_1/\pi_2)}$

where V_1 = volume at osmotic pressure of π_1.

V_2 = volume at osmotic pressure of π_2.

A value for water content of between 72% and 74% of total cell volume was obtained.

Radioactive fluxes and Ion contents

The method of using cell monolayers to measure fluxes and ion contents eliminates many of the difficulties and inaccuracies inherent in experiments which involve whole tissue preparations or cell suspensions. Intact tissue may contain several cell types, with different functional capacities. Using whole tissue, an accurate determination of extracellular space is of critical importance for correct assessment of intracellular space and contents. Generally the inulin space (using (^3H) inulin) is determined at the same time as fluxes are measured. Monolayer experiments do not involve extracellular space.

The fact that monolayers are easily and rapidly washed (x4 in 20 seconds) means that influxes can be measured accurately over short periods of time. Cells grown and used in suspension involve experimental procedures which imply two additional sources of error (Heinz, 1954); that is, back-diffusion of intracellular activity during centrifugation and radioactive contamination due to adherent medium in the packed cell pellet. Trzeciak has recorded that, after top speed centrifugation in a clinical laboratory centrifuge for 10min, there could be as much as 7% of the supernatant liquid left trapped amongst red blood cells, (Trzeciak, Krouse & Judson, 1967).

Measurement of glycine uptake

Summary Plates of cells were incubated for increasing periods of time in tritiated glycine solution and then washed in ice-cold, glycine-free solution to remove extracellular activity. The radioactive content of the cells was measured and the molarity of intracellular glycine calculated by comparison with a radioactive standard. Influx was taken as the initial uptake of (2-^{3}H) glycine over a 5min incubation time. The outward movement of radioactively labelled glycine was assumed to be relatively insignificant during this time. Different external concentrations of glycine were obtained by adding (2-^{3}H) glycine source (glycine*) (1mCi/ml) to unlabelled glycine to give a final concentration of 0.5 or 1μCi/ml. This had a negligible effect on the molarity of the solution. The specific activity of the source varied between 1.35 to 3.8 Ci/mmole.

Flux measurement methods are similar to those described by Lamb (1971) and are based on those devised by Keynes & Lewis (1951).

Detailed experimental method

(a) Plates of HeLa cells incubated at 37^{o}C in glycine*, made up in Krebs. For Xenopus cells, glycine* was

made up in W&Q salt solution and incubation was at 26°C or room temperature. For Na or K-free soak conditions, the plates were washed x2 in modified salt solution prior to addition of glycine*.

(b) After the required soak time, each plate washed x4 with ice-cold salt solution. For each wash, Krebs or W&Q was directed onto the edge of the plate, agitated, poured off and the plate drained using a suction pipette. 4 washes were normally complete within 20 seconds.

(c) Cell monolayer dispersed by addition of 1ml trypsin (0.25%). Trypsin neutralised by adding 4ml Krebs or W&Q (1% serum).

(d) Cell groups split by blasting with a syringe. 1ml of the 5ml suspension was withdrawn using an automatic pipette and placed, with 10ml liquid scintillator (NE 250), in a vial for radioactive counting in a liquid scintillation spectrometer (Packard, Tri-Carb, Model 3320). 1ml was added to 9ml ISOTON for cell counting. Each vial was mixed, using a 'Rotamixer', for 10sec before counting.

Blank plates were run, concurrently, to estimate radioactivity remaining on the plates after washing and used to obtain the background of the counter. When necessary, counts were corrected for quenching. Results were calculated on an Olivetti programma 101, or P 602. Glycine content was expressed as mmole/l intracellular water and influx as mmole/l ICW.min.

Measurement of glycine efflux

(i) Multiplate efflux

(a) Plates were incubated in Krebs (or W&Q) containing glycine* until internal glycine content was steady (approximately 1hour). At this time, the influx and efflux of labelled glycine were equal.

(b) Plates washed x4 with cold solution to remove extracellular activity, rinsed with warm solution and 10ml inactive Krebs (or W&Q) added.

(c) Inactive medium left on plates for increasing periods of time. The plates were then washed in ice-cold salt solution.

(d) Cell monolayer was trypsinised and cell groups broken up as for influx measurements. Cell numbers and radioactivity measured as previously described. The half-time of efflux could then be calculated from a semi-log plot of content against time.

(ii) Single plate efflux

This involves measuring the radioactive content of effluent rather than the cells.

(a) The plate was loaded with glycine*, as for a multiplate efflux, or loaded during the hypotonic phase of 'lysis'.

(b) After draining, the plate was washed as before and 10ml inactive Krebs (or W&Q) added. The plate was stirred by placing it on a tilted rotating kymograph drum.

(c) After 2 or 5min, a 1ml sample was collected, the plate drained and a further 10ml salt solution added. The sample was placed in a vial with 10ml scintillator for radioactive counting.

(d) The procedure was repeated and, at the end of the required efflux period, the plate was washed with ice-cold salt solution.

(e) The cells were trypsinised and samples taken for cell number, cell volume and remaining radioactive content.

In this manner, fractional loss per constant time interval could be measured. For a single intracellular compartment and exponential efflux, this

fractional loss is constant. If treatment, or a factor added to the system, affects efflux then the change can be easily detected on a histogram plot of cpm^2 against time. Alternatively, internal content of glycine can be calculated and the results expressed as half-time of efflux, or an efflux rate (mmole/lICW. min). Efflux rate = $\frac{0.693}{T\frac{1}{2}} .(S)_i$, where $(S)_i$ is initial internal concentration.

Determination of intracellular ion concentrations

Cell monolayers were washed with ice-cold Ca-sorbitol solution to remove extracellular Na^+ and K^+. Ions were extracted by lysing the cells in either distilled water or a dilute lithium standard (for Na and K) or in a dilute K standard (for Li determinations). Na and K concentrations were measured by comparison with appropriate standards on an EEL flame photometer, or an EEL 450 flame photometer, and an internal Li standard. Li concentrations were measured using the EEL 450 with an internal K standard. Cell number and volume were measured as previously described on separate, but similarly treated, plates and ion contents expressed as mmole/l intracellular water.

'Lysing' and refilling cells

Initially the technique used was that for refilling L-cells, (Lamb & Lindsay, 1971). It was found to be unsatisfactory for the following reasons:-

(a) About 35% of cells were lost from the plates during the procedure.
(b) The volume of K-filled cells was only 75% of the control.
(c) When incubated in high Na medium, after refilling, cells were unable to retain a low internal Na.

The technique was modified to give the following method:-

1) Xenopus or HeLa cells were grown to monolayers on plastic Petri dishes.

Table 5

Treatment	Cell volume (10^{-3} I.C.W.)	T-test with control
control	2.24 ± 0.09 (13)	
'lysed', reconstituted		
NaCl	2.12 ± 0.15 (6)	$p > 0.4$
KCl	2.20 ± 0.11 (10)	$p > 0.5$
choline Cl	2.20 ± 0.09 (11)	$p > 0.5$

Cell volumes of control and refilled Xenopus cells.

Cells 'lysed' 10min and reconstituted 10min with ions shown. Volumes measured using micrometer eyepiece or a Coulter channelyser. Numbers of experiments given in brackets. Values are means ± SE.

Table 6

Treatment	ion contents (mmole/l ICW)		T-test with control	
	Na	K	Na	K
control (11)	13 ± 3	132 ± 12		
'lysed' + reconstituted				
NaCl (8)	35 ± 3	78 ± 4	$p < 0.001$	$p < 0.01$
KCl (13)	12 ± 2	174 ± 12	$p > 0.5$	$p < 0.05$
choline Cl (15)	10 ± 2	64 ± 5	$p > 0.2$	$p < 0.001$

Ion contents of control and refilled Xenopus cells.

Cells 'lysed' 10min and reconstituted 10min with the chloride cation shown. Number of observations given in brackets, with mean ± SE.

2) The plates were drained, rinsed with salt solution and 'lysing' solution added (8ml per 9cm diameter plate). The plates were incubated at 27°C or room temperature (Xenopus), or at 37°C (HeLa), for 5 or 10min.
3) Cells were reconstituted by adding 0.7ml Molar chloride (K, Na, or choline) and incubating for a further 5 or 10min.

ATP or glycine was introduced during the hypotonic stage. 10min time intervals were used to obtain higher alterations in internal ion levels. The 'lysing' solution contained NaCl 10mM, $MgCl_2$ 1.2mM, cysteine (D, hydrochloride) 1mM, NaH_2PO_4 1mM, and foetal bovine serum 2.5%. The pH was 7.6 for Xenopus and 7.4 for HeLa.

General characteristics of refilled cells

Cell numbers. There was no significant loss of cells, either Xenopus or HeLa, during the procedure. The refilled cells were able to divide but 24 hours after 'lysis', cell numbers were less than on control plates; for example, in one experiment (Xenopus), control cell numbers per plate increased by 48%, whilst K-filled cells increased by 30%. (This represented a 10% difference in cell numbers). In one experiment with HeLa, 'lysis' resulted in a 6% reduction ($P < 0.02$) in cell numbers, compared with control, 24 hours after treatment.

Cell volume. Cell volumes were measured after refilling, either immediately, or after short times of incubation. Table 5 shows that Xenopus cell volumes were not significantly altered by the treatment and were not dependent upon the reconstituting cation.

Ion contents. These were significantly altered by varying the chloride ion during reconstitution. Table 6 shows ion levels immediately after the procedure was complete. Comparing with control levels, reconstituting with NaCl produced cells with a high internal

Fig.1.

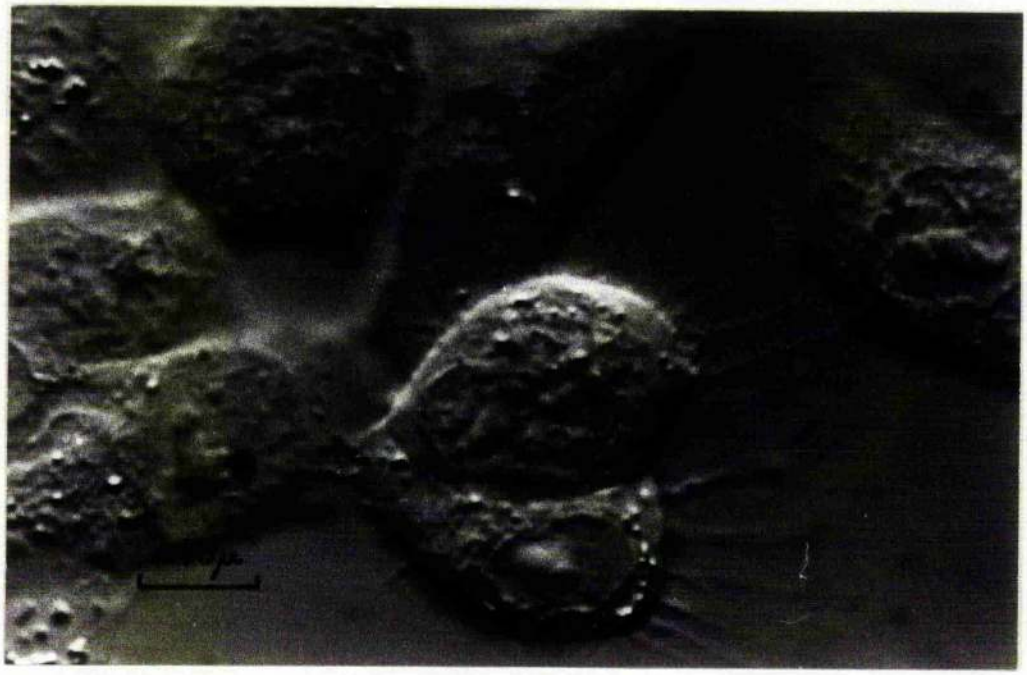
Serial observations of a group of HeLa cells to show changes recorded during the 'lysis' and reconstituting procedure. Cells reconstituted by adding molar KCl after 5min hypotonic treatment. Observed and photographed using Nomarski differential interference-contrast microscopy. Focus was on the cell periphery.

A Normal cells, in B.M.E., at room temperature.
B 1.5min after 'lysing' begun.
C After 4.5min 'lysis'.
D After 1min of reconstitution.
E After 5min of reconstitution.

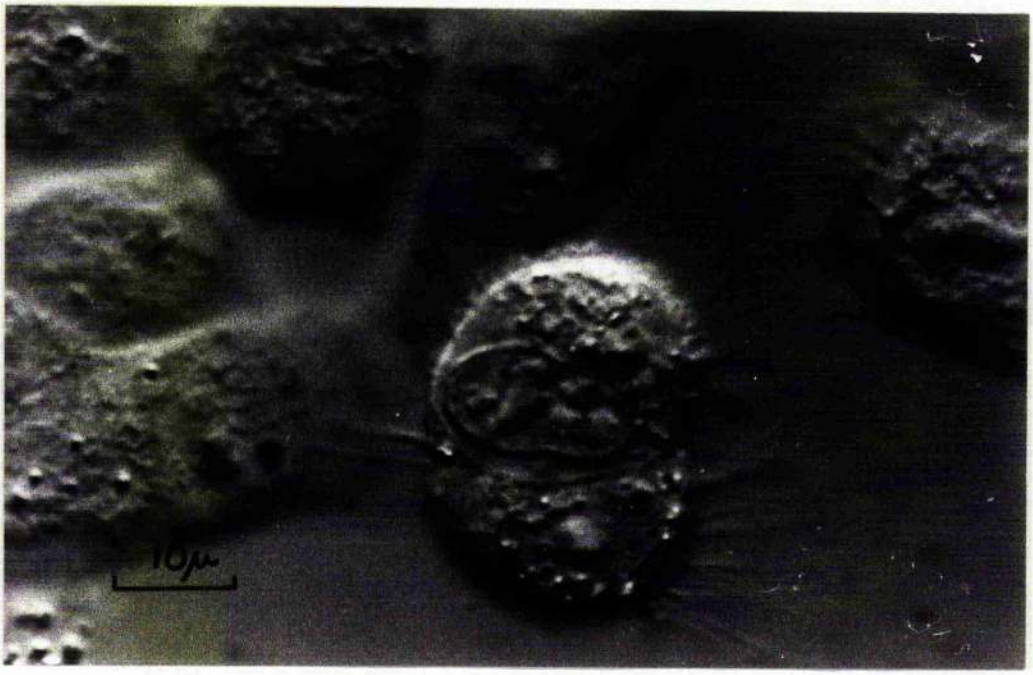
A

B

C



D



E

Na and low K; with KCl, a high K; with choline chloride, a low K. Reconstituting with K or choline did not significantly alter internal Na. Intermediate values were obtained by using different proportions of Na, K and choline during reconstitution of the cells.

Cell appearance. Xenopus and HeLa cells were observed during the hypotonic and reconstituting stages of the procedure using Nomarski differential-interference-contrast microscopy (Fig.1). The cells were grown to a monolayer on glass coverslips and the coverslips fitted with a grease seal over a hole in the bottom surface of a Petri dish. A single cell could then be observed whilst the surrounding fluid was changed.

(1) 'lysis'. All HeLa and Xenopus cells observed swelled. This was indicated by an increase in diameter and depth. Most of the swelling occurred within 1min of 'lysing' and was followed by nuclear swelling. The increase in peripheral outline observed during 1 to 5mins of 'lysing' may reflect this nuclear increase. Xenopus cells are characterised by a large number of filopodia-type extensions. 5mins of 'lysis' results in reorganisation of these extensions into single swellings with no visible internal features. The 'filopodia' are distinct from cellular processes which occur in both cell types and form intercellular contacts. The latter also appear swollen but retain their integrity. Brownian movement was observed in the cell. No outflow of cellular content was seen.

(2) reconstitution. The cells, nuclei and cellular extensions shrank immediately on addition of the chloride solution. Filopodia reappeared although they were generally reduced in number. Often a 'rounding-up' of the cells was observed but the volume after 5mins of reconstituting was approximately that of the original cell. The cell and nuclear outlines were slightly altered

by the procedure. In Xenopus cells an additional feature was observed; at 1min of reconstituting, large regular-shaped vacuoles were occasionally observed within the cell outline; these disappeared within 5mins but were thought to represent shrinkage of the cells from the coverslip contact, possibly indicating areas of restricted diffusion.

Suspensions of mammalian erythrocytes have been haemolysed by hypotonic treatment, (Hoffman, Tosteson & Whittam, 1960; Whittam & Ager, 1964; Lant & Whittam, 1968). The ghosts produced were spherical but shrank, to become crenated, when the osmolarity of the haemolysate was restored to that of plasma by addition of alkali-metal chloride. The volume of the reconstituted ghosts was only $\frac{1}{4}$ to $\frac{1}{3}$ of the volume of the original erythrocytes and the haemoglobin 18% normal. After reconstituting, up to 40min incubation at 37°C in 150mM K increased the K content (to 60mM) and allowed the ghosts to regain a low K permeability. Ion contents could be varied as they were determined by the alkali-metal chloride added to the haemolysate. Similar techniques have been applied to pigeon red cells suspensions, (Vidaver, 1964), and L cell monolayers, (Lamb & Lindsay, 1971). In pigeon cells, by varying the proportions of Na and K during the treatment, internal Na could be varied between 24 and 126, where internal Na + K = 152mmole/l ICW. The restored cells had a limited ability to maintain a high internal K and low Na, cells losing K at a rate of 32% per 40min, independent of the outward K gradient. The cells were then used to measure glycine transport, assuming that results would be qualitatively similar to those in intact cells. In the L-cell experiments, cells were lysed 10min and reconstituted with K for 3min. The refilled cells were more leaky than normal but appeared to be viable in that the efflux of ^{45}Ca from refilled cells was similar to that from normal cells and was dependent upon ATP.

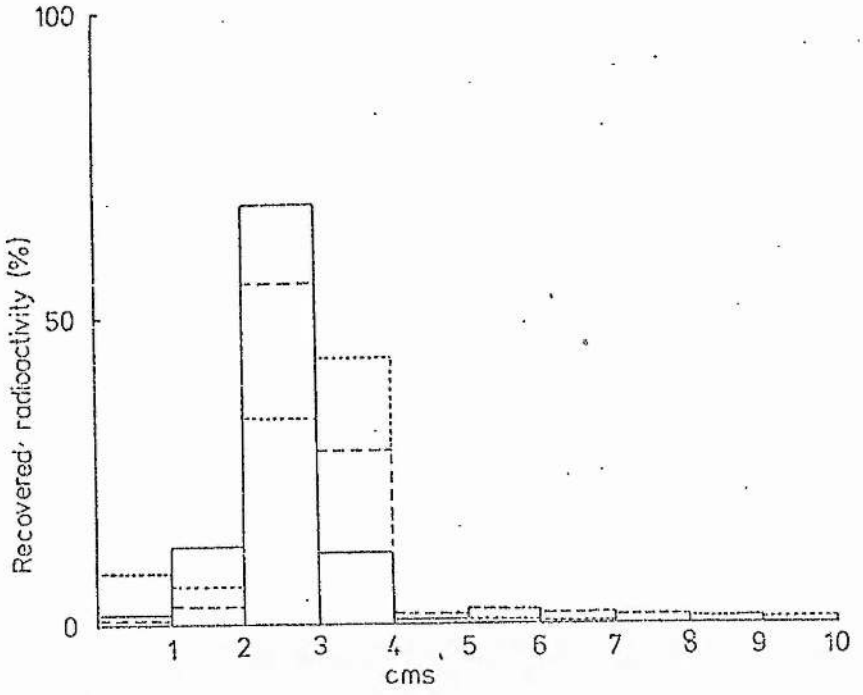


Fig.2. Radiochromatogram of (^{3}H) glycine. Glass plates coated with silica gel to give a thin layer 0.3mm thick, air dried and activated during ½h at 105°C. 0.1ml aliquots of standard or cell extract applied at 0 and run for approximately 3h, until the solvent had migrated 10cms. Plates air dried and the silica divided into 1cm bands for radioactive counting. Extracts prepared from HeLa cells incubated at 37°C for 5min ——— or 80min – – – –, in Krebs containing ^{3}H glycine + unlabelled glycine to give a concentration of 1mM. The external soak solution was used as the standard ····· . By analysis of variance there was no significant difference between water and solvent extractions for either influx ($P > 0.75$), or accumulation ($P > 0.25$), and no significance between influx and accumulation fractions with either water ($P > 0.25$) or solvent ($P > 0.75$) extraction procedures. The percent error on the radioactive counts was calculated to be between ±2 and ±5%, (Glenn & Lamb, 1971).

Chromatography

The labelled compound within the cell was demonstrated to be glycine, rather than some metabolic product, by radiochromatography.

Monolayers of HeLa cells were incubated at 37°C for 5 or 80min with (2-^{3}H) glycine, added to unlabelled glycine at an external concentration of 1mM. After incubation, the cells were washed 5 times and immediately extracted with solvent, (n-butanol:water: acetic acid). The extract was centrifuged at 1800g for 15min and the supernatant chromatographed on a thin layer of silica gel using n-butanol:water:acetic acid (12:5:3), as the solvent. A standard of (2-^{3}H) glycine, from the incubation medium, was run simultaneously. Fig.2 shows that 81% of the standard radioactive label was collected between 2 and 4cm, compared with 84% from the 5min influx and 78% from the 80min accumulation. With a similar solvent, the R_f value for glycine is 0.22, (Randerath, 1964). This demonstrated that the tritium measured during influx experiments was still on the glycine molecule but that there was possibly a small loss of label during accumulation.

Kinetic Analyses

Data was collected for influx of (^{3}H) glycine at concentrations of external glycine varying from between 0.05 and 5mM, in several experiments. (^{3}H) glycine was added to different concentrations of unlabelled glycine to give 0.5μCi/ml. The data was used to check whether the influx results conformed to a first-order model and to determine Vmax and the apparent Km.

The Michaelis-Menten equation for enzyme kinetics, which relates velocity of reaction (v) of an enzyme with a substrate and the substrate concentration (s), may be written as

$$v = (s/s+Km).Vmax$$

with the kinetic constants Vmax (v, when $s \rightarrow \infty$) and Km (s, when $v = Vmax/2$).
The equation has 3 linear transformations :-

$$(1)\quad \frac{1}{v} = \frac{1}{Vmax} + \frac{Km}{Vmax} \cdot \frac{1}{s}$$

$$(2)\quad \frac{s}{v} = \frac{Km}{Vmax} + \frac{s}{Vmax}$$

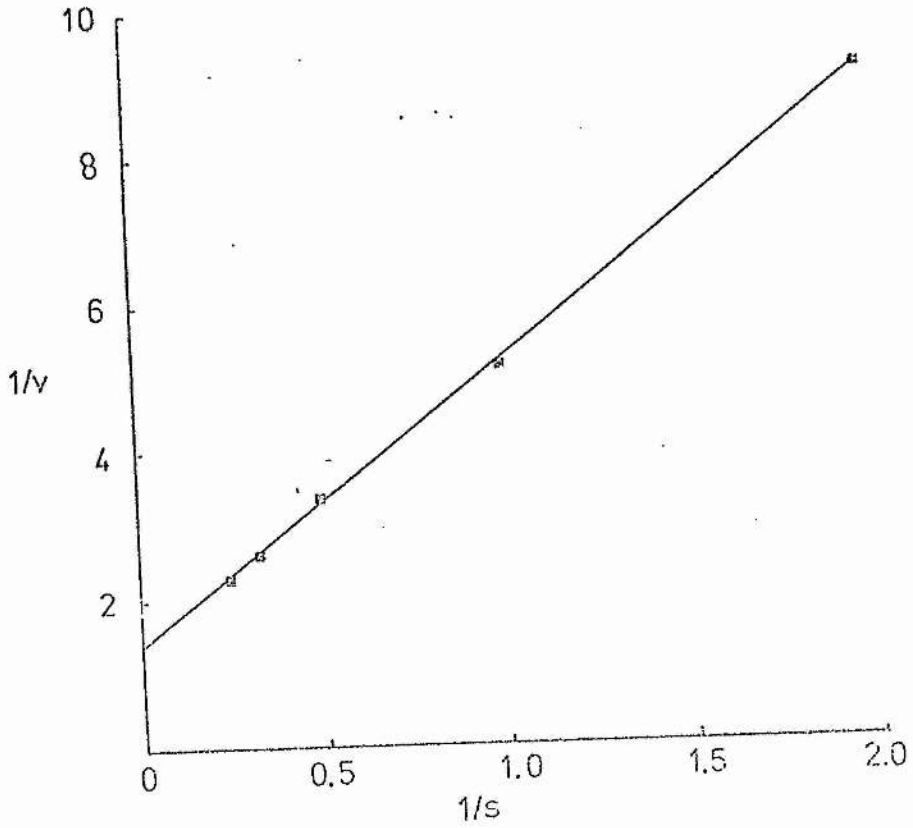
$$(3)\quad v = Vmax - Km.\frac{v}{s}$$

Estimates of kinetic constants can then be obtained graphically or statistically from the variables (1) $1/v : 1/s$, (2) $s/v : s$, (3) $v : v/s$.

A certain amount of controversy exists as to the best method although generally the double reciprocal (Lineweaver and Burk) plot is used. For example, in 1971, 97.3% of transformed plots in J. of Biol.Chem. were $1/v : 1/s$ (Walter, 1974). This plot is preferred by Dixon and Webb (1959) in that (a) values of v can be readily identified with particular substrate concentrations (b) in the Eadie plot of $v : v/s$, any error in v will affect both coordinates and displace the point obliquely (c) deviations from linearity are revealed almost equally well by the Lineweaver & Burk and Eadie plots and (d) with respect to the accuracy of the constants determined, the methods are about equally good.

In principle, any linear plot can be used to distinguish whether data fit a classical first-order model or a model which involves more than one binding site. If the system is a second-order model (the simplest alternative), then it appears that curvature is most obvious in a $v : v/s$ plot for any finite substrate concentrations, (Walter, 1974). Plots of $1/v : 1/s$ and $s/v : s$ tend to make a second-order model appear as a first-order. Using the $1/v : 1/s$ plot there is a tendency to 'obscure deviations from linearity' (Hofstee, 1959) as the significance of values of v at low values of s is overemphasised and any deviation from linearity at higher substrate concentrations

3(a)



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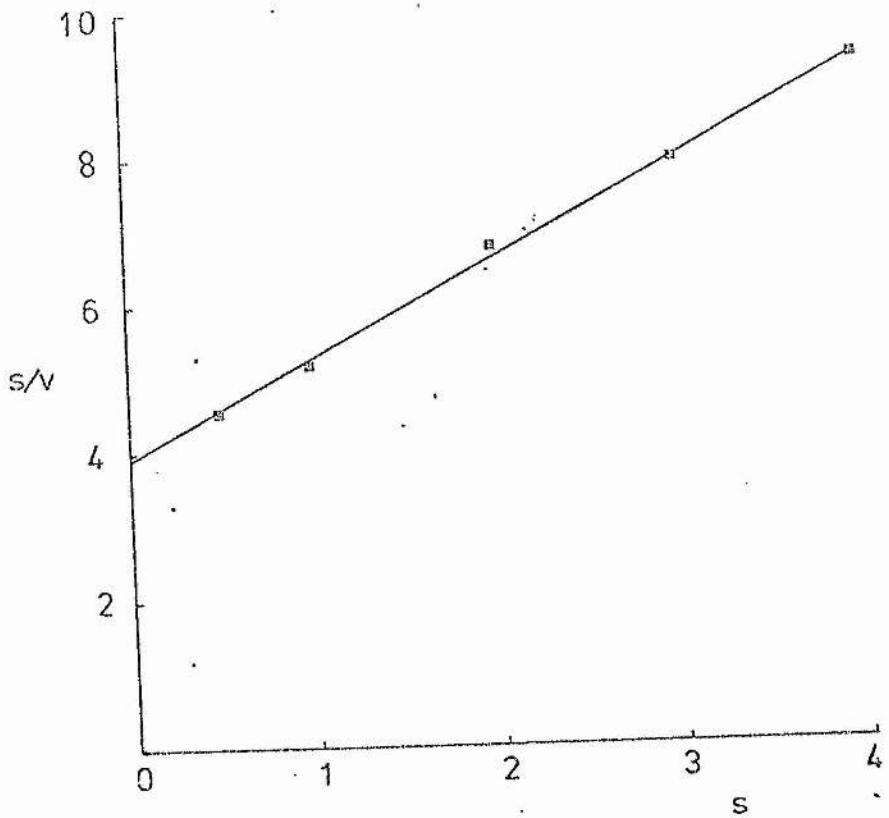
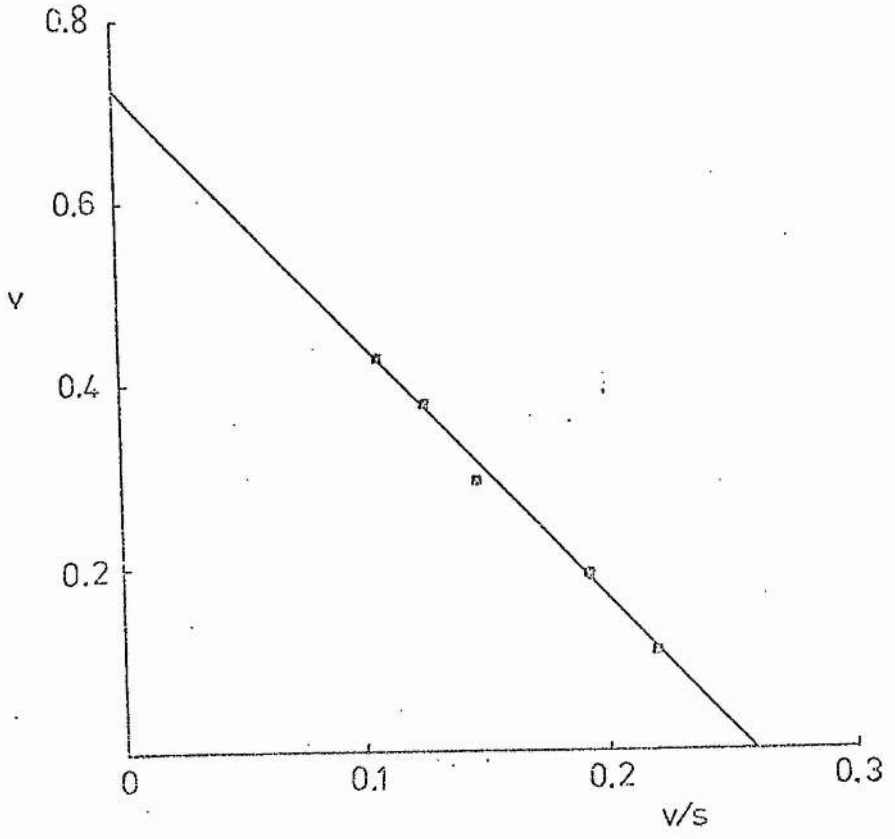


3(c)



Kinetic analyses (captions to graphs).

Fig.3. Dependence of glycine influx (v), expressed as mmole/lICW.min, on extracellular concentration of glycine (s), expressed as mM, in Xenopus cells. Influx measured by 5min incubation at room temperature in varying concentrations of (3H) glycine in W&Q salt solution. Lines drawn by regression analysis.

(a) 1/v : 1/s plot of the data, r = 0.9998. Vmax (from the reciprocal of the ordinate intercept) = 0.72 mmole/lICW.min. Km (from slope x Vmax) = 2.77mM.

(b) s/v : s plot of the data, r = 0.9988. Vmax. (from the reciprocal of the slope) = 0.74 mmole/ lICW.min. Km (from ordinate intercept x Vmax) = 2.86mM.

(c) v : v/s plot of the data, r = -0.9971. Vmax (from ordinate intercept) = 0.73 mmole/lICW.min. Km (from -slope) = 2.81mM.

becomes less obvious, (Coleman, 1965). Thus, these writers would support the use of an Eadie plot (v : v/s).

If Km and Vmax are estimated, using correct weights for the data, identical values will be obtained by statistical analysis of the various plots but deviations from correct weighting will result in less accurate values for Km and Vmax. Assuming constant variance in values of v, then, over a range of substrate concentrations from $\frac{1}{3}$ to 3x Km, the relative weight (1/variance) of 1/v varies by a factor of 80 whereas the relative weight of s/v varies by less than a factor of 2, (Wilkinson, 1961). It follows, therefore, that with unweighted data, over practical ranges of substrate concentration, the linear plot of s/v : s is preferable to the Lineweaver & Burk plot.

Figs. 3a, 3b and 3c show plots of 1/v : 1/s, s/v : s and v : v/s, respectively, for the data of glycine influx (v) at varying external glycine concentrations (s) shown in Fig.7 of the results. By regression analysis, the data gave significant linear correlations ($P < 0.001$ in each instance) although the points were more evenly distributed in the s/v : s plot because of the substrate concentrations chosen. Estimated values for Vmax and Km are given in the captions for each graph and are similar for all 3 plots. When the calculated kinetic constants were incorporated into the Michaelis equation and used to fit a theoretical curve to the data, those derived from the Lineweaver & Burk plot gave a curve which fitted less well the influx values obtained at the higher substrate concentrations.

In view of this and the arguments outlined above, the kinetic constants were generally calculated by regression analysis of s/v : s of the experimental data. Data were expressed graphically as well as analysed statistically for significance levels, to check more easily for deviations from a single curve.

Although there was considerable variation in results obtained from different experiments, the values obtained within individual experiments were consistent. For comparison of results obtained under differing experimental conditions, a control was run concurrently. Because of the type of error associated with a particular value of Vmax and its related Km (Colquhoun, 1971, Fig. 12.8.2(a)) when an apparently significant change in parameters was induced by treatment, the results were taken to be meaningful only if they did not involve change in both parameters in the same direction.

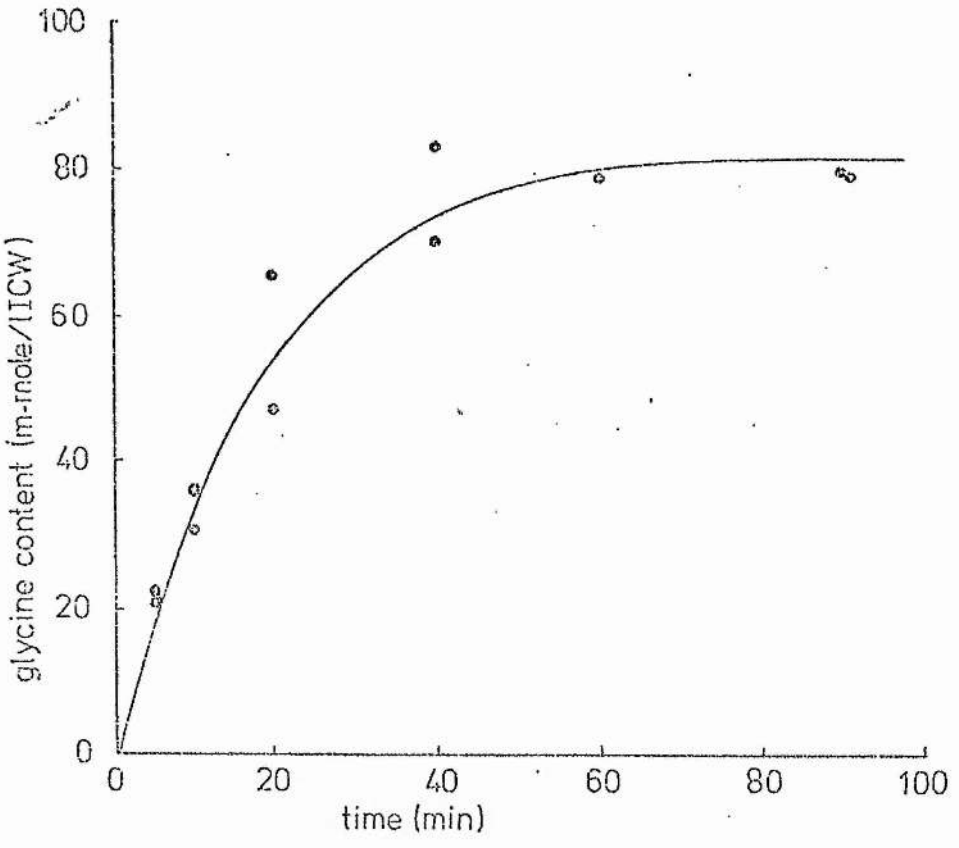


Fig.4. Time course of glycine uptake into HeLa cells. Incubation at 37°C, with 10mls Krebs containing 2mM (^{3}H) glycine, for the times shown on the abscissa. Each point is a single observation, in one experiment. Line drawn by eye.

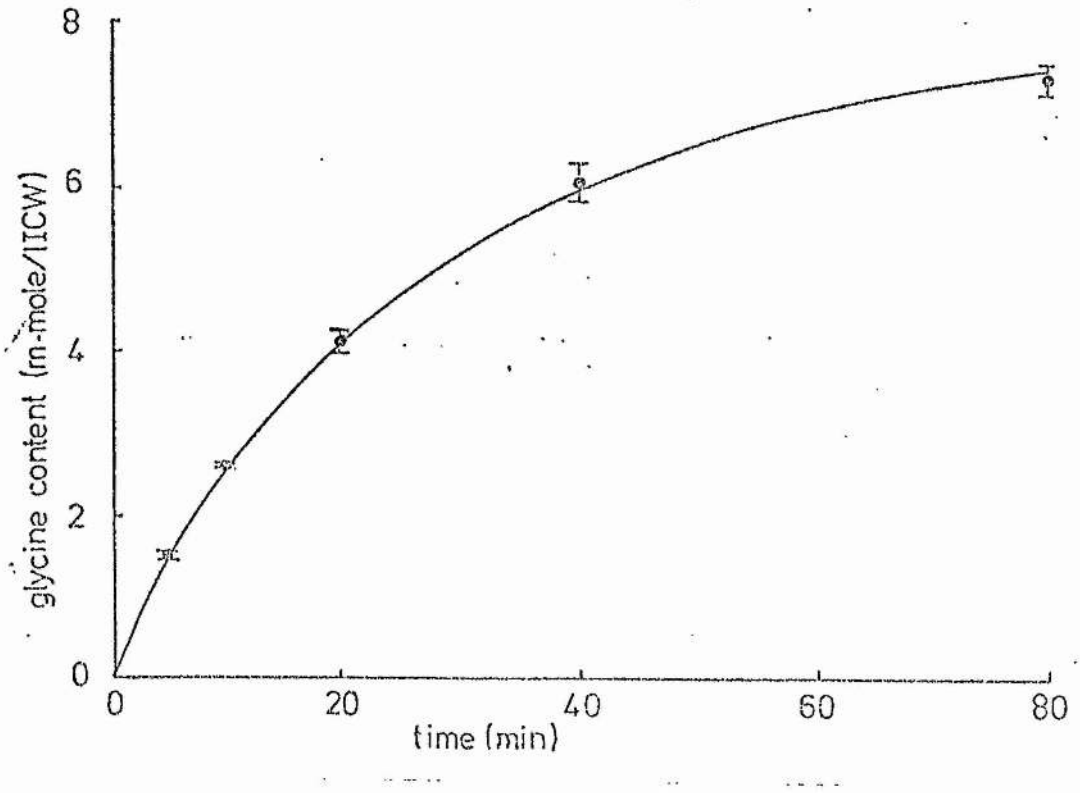


Fig.5. Time course of glycine uptake by Xenopus cells. Incubation at room temperature in 10mls W&Q salt solution containing 2mM (^{3}H) glycine, for the times indicated. Each point represents the mean of 4 readings $\pm$ S.E. Line drawn by eye.

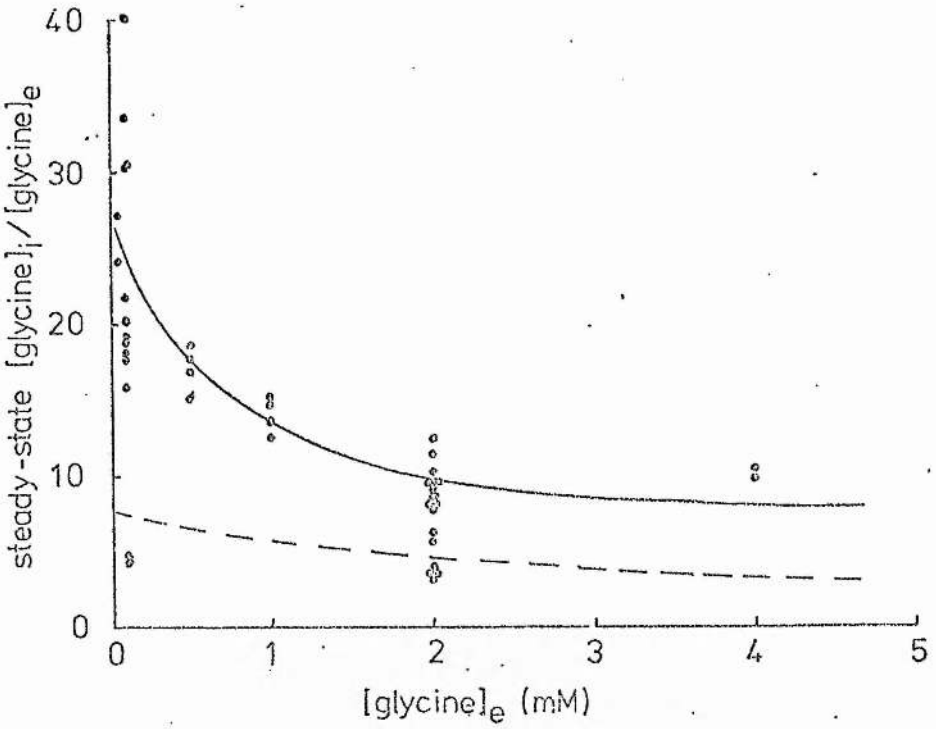


Fig.6. Effect of external glycine concentration on glycine accumulation in Xenopus cells. Incubation at room temperature for between 60 and 90min in W&Q salt solution containing the concentration of (2-^{3}H) glycine given on the abscissa. Each point represents an individual reading from 13 experiments. Solid line drawn by eye, to fit data. Broken line drawn to fit a proposed mechanism of transport, (see discussion), with kinetic constants, Km^o = 2.37mM, $Vmax^o$ = 0.88mmole/lICW. min, Km^i = 83.8mmole/lICW, $Vmax^i$ = 4.00mmole/lICW.min, (i.e. mean values obtained experimentally).

RESULTS

Characterisation of transport system

Accumulation

Figs.4 and 5 show the rates of uptake and accumulation of glycine into HeLa and Xenopus cells, respectively. Values were consistent within each experiment but there was inter-experimental variation, so that results shown are from 'typical' experiments. The entry rate of glycine was linear over the first 5min but levelled to give a final distribution ratio between intra- and extra-cellular water, at 60 to 90min. This indicated that there was a steady-state, at which influx and efflux were equal. The distribution ratio decreased as extracellular glycine concentration increased, (see Fig.6).

The values for accumulation (of approximately 8 in Xenopus and 40 in HeLa, for an external glycine of 2mM) are of the same order as those found by other workers in different preparations. In Ehrlich cells, using an external glycine of 1mM, 60min incubation at 37°C gave a final distribution ratio of 15, (Oxender & Christensen, 1963). In pigeon erythrocytes, glycine accumulation ratios of 20-40 were obtained, (Terry & Vidaver, 1973). Glycine accumulation values were also consistent with distribution ratios found for the non-utilisable amino acid analogue, AIB, (Kuchler & Marlowe-Kuchler, 1965). External concentrations ranged from 0.1mM in cultured human fibroblast lines, giving a final distribution ratio of between 10 and 25 in 60min (Mahoney & Rosenberg, 1970) to 5mM in immature rat uterus, to give a 7-fold distribution ratio, (Riggs & Pan, 1972).

Influx

More information about the amino acid transfer process was obtained by studying influx and efflux. Influx was taken as the initial rate of uptake of (2^3H)glycine.

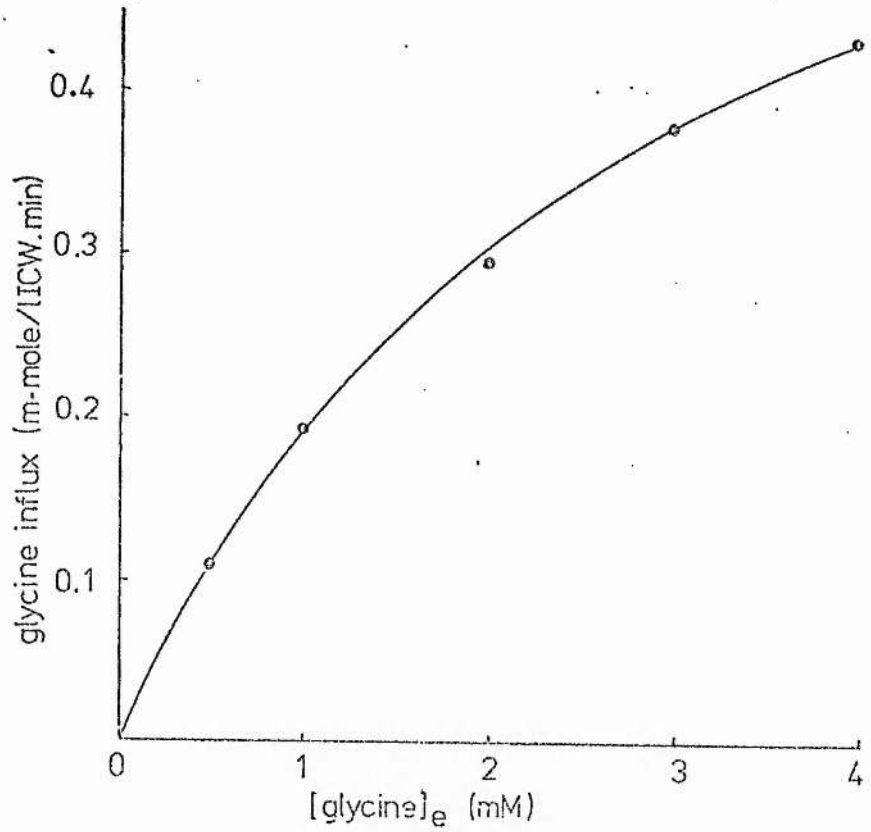


Fig.7. Influx of (^{3}H) glycine in Xenopus at the concentrations of external glycine shown on the abscissa. Influx measured over 5min at room temperature. Each point represents an individual reading. Line drawn to fit Michaelis equation, with the parameters Vmax = 0.74mmole/1ICW.min, Km = 2.86mM. (calculated from S/V : S plot of the data). The different external concentrations were prepared by adding (^{3}H) glycine (specific activity of 2.2 Ci/mmole) to unlabelled glycine to give a final concentration of 0.5 μCi/ml.

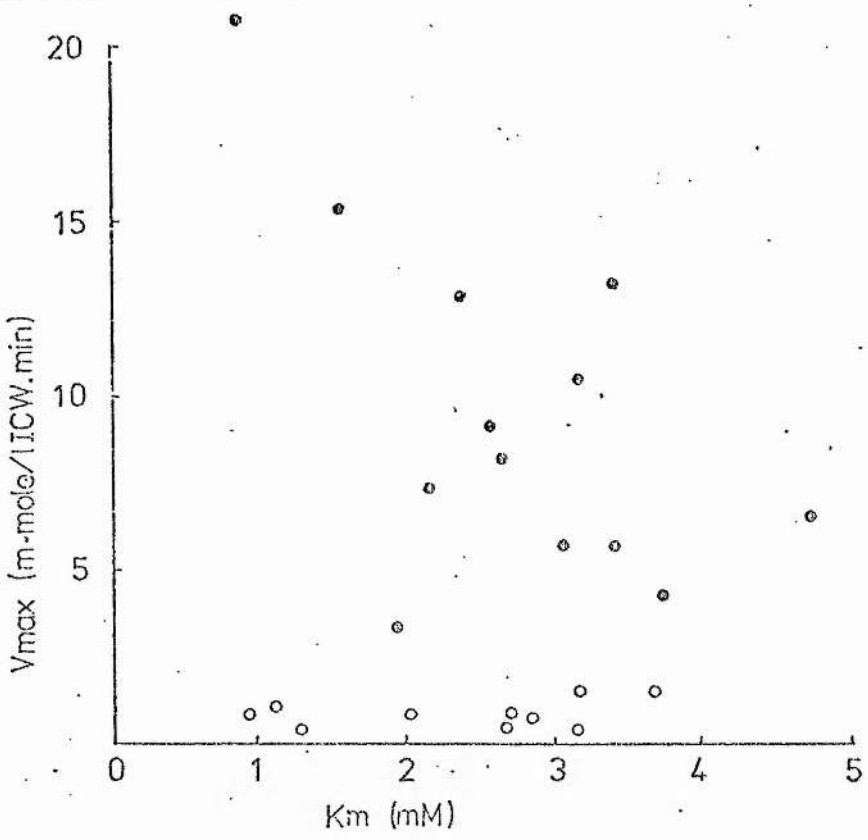


Fig.8. Vmax plotted against apparent Km values for glycine influx in HeLa (●) and Xenopus (o) cells. Each point represents one experiment and was calculated from plots of S/V : S of the experimental data. Cells grown 3 to 4 days in normal media and influx measured over 5min at 27°C or room temperature (Xenopus), or 37°C (HeLa).

Fig.7 shows influx of glycine in Xenopus cells as extracellular glycine concentration was varied. As the glycine concentration was increased the relative influx decreased. This was not in agreement with Fick's first law of diffusion and indicated that glycine entered the cell by restricted diffusion, if it was assumed that the amino acid did not combine with a cellular substance whose capacity was limited during the time of influx. The data fit the Michaelis-Menten equation for enzyme kinetics, (see method). Similar curves have been obtained using external glycine concentrations in the range 0.05 to 5mM. The single component indicated that influx was mediated by a single transport system.

Qualitatively similar results were obtained for HeLa cells. Fig.8 gives Vmax and associated apparent Km values obtained from several experiments and shows the experimental variation. Mean ($\pm$SE) values were:- for Xenopus, Vmax = 0.88 ($\pm$ 0.13)mmole/lICW.min, Km = 2.37 ($\pm$ 0.30)mM; for HeLa, Vmax = 9.48 ($\pm$ 1.38)mmole/lICW.min, Km = 2.76 ($\pm$ 0.28)mM. By regression analysis, there was no correlation between Vmax, Km, cell volume or cell number per plate, in either cell type. The cause of the variation was not apparent.

Apparent Km values of the same order have been recorded in several cell and tissue types; eg., in Ehrlich cells, Km = 3.7mM (Heinz, 1954), 6.4mM, (Scholefield, 1961); in mouse pancreas, Km = 6.7mM (Lin & Johnstone, 1971); in mouse ascites tumour cells, Km = 5mM (Eddy, Mulcahy & Thomson, 1967).

Efflux

Using the 'lysis' technique, cells could be loaded with (2-^{3}H) glycine during the hypotonic stage, reconstituted and the efflux measured using a single plate to obtain sequential radioactive fractional losses. Typical data are presented in Fig.16 (i) and (ii).

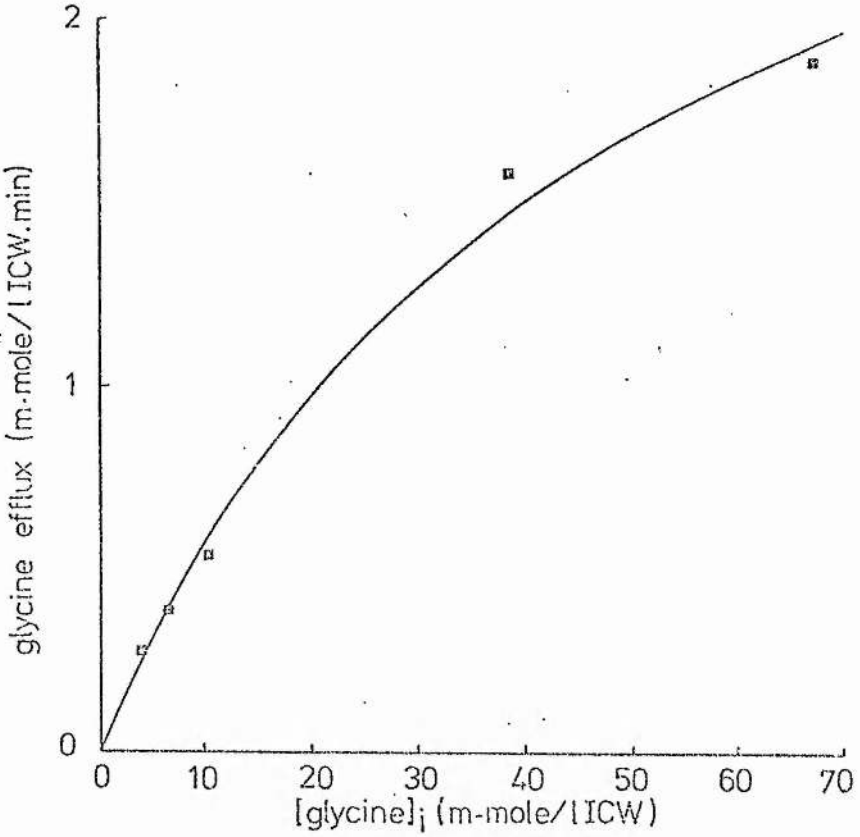


Fig.9.

Efflux of glycine* from Xenopus cells

Cells 'lysed' 10min, room temperature, in the presence of varying (2-^{3}H) glycine concentrations and reconstituted 10min by adding choline Cl. Single-plate efflux procedure gave fractional loss per 3min time interval, (room temperature). External glycine* concentrations calculated by direct comparison with a known radioactive standard. Cell volume and cell number were not affected by treatment. Internal K was approximately 45mmole/l ICW, internal Na was approximately 7mmole/lICW. Line drawn to fit Michaelis-Menten kinetics, with kinetic constants calculated from S/V : S plot of the data. Each point is calculated from 6 fractional loss values, obtained from one plate of cells.

All normal effluxes measured gave fractional losses which were independent of the time after efflux was begun; indicating a single exponential loss. Semi-log plots of intracellular glycine* content against time were correlated by regression analysis and gave linear relationships. The half-time, the rate constant and the rate of efflux could then be calculated.

Fig.9 shows the result of one experiment in which efflux was measured, varying the initial internal glycine* concentration by varying the external concentration during 'lysis'. The efflux rate was not linearly related to initial internal concentration but became saturated. The relationship obeyed Michaelis-Menten kinetics; giving, in this instance, values for Vmax = 3.29mmole/lICW.min, and Km = 47.7mmole/lICW. Taking all values obtained for efflux rates, mean values of Vmax = 4.00mmole/lICW.min, and Km = 83.8mmole/lICW were obtained.

No directly comparable experiments were carried out using HeLa cells.

In one 'multiplate-efflux' experiment, normal (non-lysed) Xenopus cells were loaded during 90min incubation in W&Q plus (2-^{3}H) glycine, 0.1mM. With an initial internal glycine* concentration of 1.79mmole/lICW an efflux rate of 0.14mmole/lICW.min was obtained. Using the kinetic constants calculated from 'lysing' experiments, the estimated value was 0.12mmole/lICW.min, or 0.08mmole/lICW.min.

It thus appeared that 'lysed' Xenopus cells behaved normally with respect to amino acid efflux properties. The 'lysing' technique (allowing controlled, independent variation of initial intracellular glycine and ion concentrations), could then be used for further investigations.

Exchange diffusion

It was expected that, if exchange diffusion formed a major part of the uptake system of glycine in Xenopus

Table 7

treatment	glycine influx (mmole/lICW.min)
control	0.78 ± 0.06
lysed -glycine	0.73 ± 0.02
lysed +glycine	0.71 ± 0.04

Effect of intracellular glycine concentration on glycine influx into Xenopus cells.

Cells equilibrated 30min, 27°C, in W&Q salt solution (1% serum) prior to experiment. Plates rinsed, 'lysed' 5min +/- 2mM glycine, and reconstituted with KCl for 5min. (Internal glycine content = 8 ± 1mM, from previous experiments.) Control plates left for 10min with no further treatment. Plates drained, rinsed and the influx of (^{3}H) glycine measured (5min at 27°C, from $(g)_o$ = 2mM). Analysis of variance showed no variation in cell volume or numbers per plate. By Student's T-test, when compared with the control, 'lysis' with or without glycine had no significant effect on the subsequent glycine influx, ($p > 0.2$). Each value = mean of 3 readings ± SE.

cells, influx ought to be increased if the internal concentration of amino acid were increased by pre-treatment.

Removal of growth medium and preincubation in salt solution results in a low internal amino acid pool, (Piez & Eagle, 1958). Xenopus cells were equilibrated in W&Q salt solution and, using the 'lysis' and refilling technique, were either further depleted of amino acids or loaded with unlabelled glycine. Glycine influx was subsequently measured using (2-^{3}H) glycine. As can be seen from Table 7, influx was unaffected by internal glycine concentration. It, therefore, appeared that exchange uptake of glycine did not normally occur in Xenopus cells.

The effect of external amino acid during the efflux of glycine was investigated. Cells were 'lysed' and refilled with (2-^{3}H) glycine and efflux measured by collecting the sequential fractions lost per 2 or 5min intervals. During efflux the external medium was varied by adding 5mM unlabelled glycine to W&Q salt solution. Efflux of glycine* was then into extracellular media with or without added glycine. The $(glycine)_e$ sequences were varied between plates. By analysis of variance, efflux was not significantly altered by the presence of 5mM glycine externally, ($P > 0.5$). When results were expressed as fractional loss per time interval +/- glycine, then:- Expt.1, +glycine = 0.101 $\pm$ 0.016, -glycine, = 0.108 $\pm$ 0.016, ($P > 0.5$), for 2min fractional loss. Expt.2, +glycine = 0.120 $\pm$ 0.007, -glycine = 0.125 $\pm$ 0.009, ($P > 0.5$), for 5min fractional loss. In each case the mean of 10 readings $\pm$ SE is given.

In one experiment with normal HeLa cells (ie. not lysed) cells were preloaded with glycine* by preincubating for 60min in 0.1mM (2-^{3}H) glycine in Krebs. Efflux rate was calculated from the content* remaining in the cells after various incubation times in non-radioactive Krebs +/- glycine, 10^{-4}M. The presence of this concentration

Table 8

External (K) (mM)	glycine influx (mmole/l ICW.min)
0.2	0.37 ± 0.05 (3)
2.1	0.35 ± 0.03 (2)
5.1	0.34 ± 0.05 (4)
10.0	0.28 ± 0.01 (4)
15.2	0.33 ± 0.06 (4)

Effect of external K on glycine* influx (Xenopus)

Plates rinsed in low K salt solution and glycine* uptake measured over 5min from 2mM glycine, at the concentrations of K shown (room T°C). External Na remained constant. There was no significant effect of treatment on cell numbers per plate, cell volume, or glycine influx. Values given are means ± SE, with the number of observations in brackets. The normal K was about 5mM.

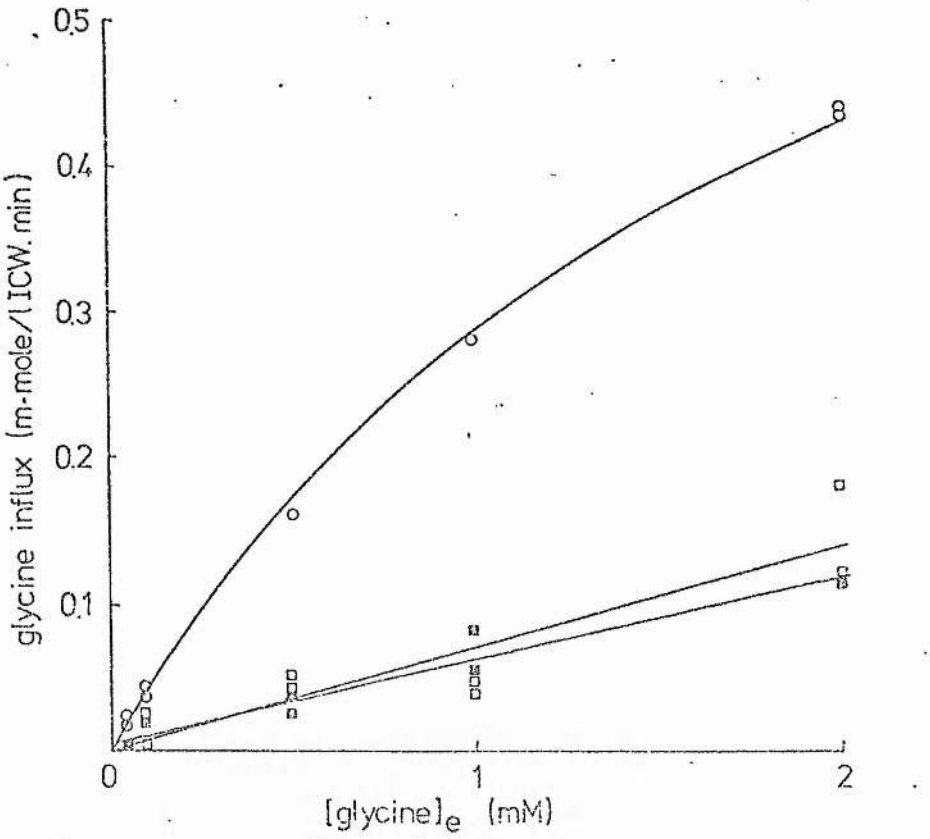


Fig.10.

Effect of total removal of external NaCl on glycine* influx (Xenopus).

Plates rinsed in appropriate Na^+ concentration and uptake measured for 2min, at room temperature, from the concentrations of glycine shown on the abscissa. NaCl was replaced in an iso-molar manner with either KCl or choline Cl, so that ion contents (mM) of soak media were: control {○}, Na = 69, K = 5; with potassium {□}, Na = 74, K = 67; with choline {■}, Na = 3, K = 5. For control influx values, the line is drawn to fit a Michaelis-Menten relationship, with kinetic constants calculated from S/V : S plot of the data (correlation of $P < 0.001$). Low-Na data did not obey M-M kinetics over the concentrations of glycine* used, but lines are drawn from linear regressions of V : S, ($P < 0.001$). The effect of replacing Na^+ with K^+ was not significantly different from the choline effect ($p > 0.2$). Cell number and volume were not affected by the treatment. Each point represents an individual reading, from 2 experiments.

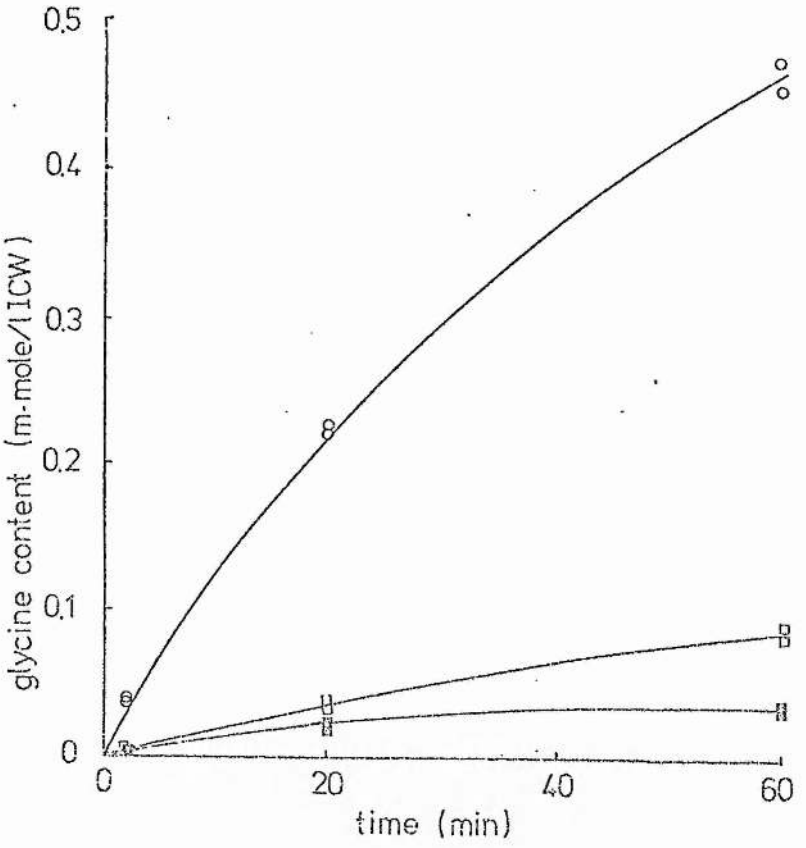


Fig.11.

Effect of total removal of external NaCl on glycine* accumulation (Xenopus).

Plates rinsed in appropriate [Na], and glycine* contents measured after incubation at room temperature for the times indicated on the abscissa. External glycine* = 0.1mM, prepared in W&Q salt solution with the following ion levels: control [o] Na = 80, K = 7; with KCl in place of NaCl [□], Na = 0.3, K = 90; with choline Cl [▣], Na = 0.2, K = 7, (concentrations expressed as mM). Cell number and volume were not affected by treatment. Each point represents an individual reading. Lines drawn by eye.

of glycine had no effect on the half-time of efflux.

Effects of ionic composition of extracellular media on glycine uptake

1. Xenopus

Potassium Table 8 shows that there was no significant effect of external K concentration (0.14 to 15.18mM) on glycine influx when the concentration was varied during the 5min incubation time. External Na remained constant at 67mM. No other ion was added to the W&Q salt solution and no adjustment was made for any change in the osmolarity of the influx medium. Thus, influx of glycine appeared unaffected by a change in external K alone.

Sodium Fig.10 shows the effect of replacing external NaCl with KCl or choline Cl during 5min influx measurements from varying external concentrations of glycine. In the soak solution containing normal ion levels, the uptake obeyed Michaelis-Menten saturation kinetics, (Vmax = 0.86mmole/lICW, Km = 2.03mM). Total replacement of NaCl in the soak solution resulted in a reduced glycine influx. Over the concentration range investigated, the data could not be fitted to Michaelis-Menten kinetics; but, by regression analysis, significant linear correlations were obtained for both choline and K replacement. The effects of choline and K were not significantly different.

This reduced influx resulted in an inability of the cells to accumulate glycine. Fig.11 shows a normal distribution ratio, and equilibration values when external NaCl was replaced by either K or choline Cl. In neither case were the cells able to concentrate glycine above the external concentration of 0.1mM. Although influx was reduced equally by choline or K replacement, the equilibrium level of intracellular glycine was greater when K was the replacing ion.

Table 9

external sodium concentration (mM)	Vmax (mmole/l ICW.min)	Km (mM)
62	0.37	2.40
10	0.30	5.45
92	0.87	0.94
72	0.88	1.47

Effect of external Na on kinetic constants of glycine* influx (Xenopus).

Glycine* influxes measured over 5min at room temperature, at varying extracellular glycine and Na concentrations, (NaCl replaced with choline Cl). Data correlated to give straight lines (by regression analysis, $P < 0.001$ or $P < 0.02$), with Eadie plots of S/V : S, where V = influx and S = external concentration of glycine. External K was not varied. Data from 2 experiments.

Table 10

Expt.	External Na concentration (mM)	Substituting chloride	Vmax (mmole/lICW.min)	Km (mM)
1.	135	-	13.25	3.43
	35	choline	13.05	6.40
	36	lithium	11.09	6.25
2.	130	-	3.37	1.95
	75	choline	2.62	1.23
	35	choline	3.05	2.93
	2	choline	3.30	14.63

Effect of external Na on kinetic constants of glycine influx (HeLa).

Glycine* influxes measured over 5min (expt.1) or 2min (expt.2), at 37°C, in Krebs with NaCl partially replaced with ions shown. Data correlated to give straight lines by linear regression analysis of Eadie plots of S/V : S. External K was constant at 6mM. Cell volume and number were not affected by treatment.

As total replacement of NaCl resulted in such a large change in the transport capacity of the system, kinetic aspects of the alteration were studied with only partial Na replacement. Usually a 20% reduction in external Na had little effect but further reduction resulted in a decreased influx for all concentrations of glycine used. Absolute values varied inter-experimentally so that analyses were carried out within experiments. In 2 experiments (analyses shown in Table 9) the apparent Km of glycine* influx increased significantly ($P < 0.05$) as external Na was reduced (indicating a lower carrier affinity) whilst Vmax was not altered significantly.

In a similar manner, data for glycine influx could be linearly correlated with external Na concentration (C) in a plot of C/V : C. This indicated a 1:1 relationship between Na and glycine for each concentration of external glycine used, (0.05-2.0mM). The affinity of the system for Na remained constant within experiments but varied inter-experimentally. Values for Km of Na were recorded between 9 and 20mM of external Na, suggesting that there were differences in the Na sensitivity of the glycine carriers between groups of cells.

2. HeLa

Sodium Results of kinetic analyses of influx values obtained at varying external glycine and sodium concentrations are shown in Table 10. Results are qualitatively similar to those found for Xenopus cells in that reduction in external Na resulted in a significantly increased Km of influx ($P < 0.001$) with no significant change in the Vmax (indicating a decreased carrier affinity for amino acid with no alteration in the carrier number or rate of turnover). There was no significant difference between choline and lithium replacement of external sodium.

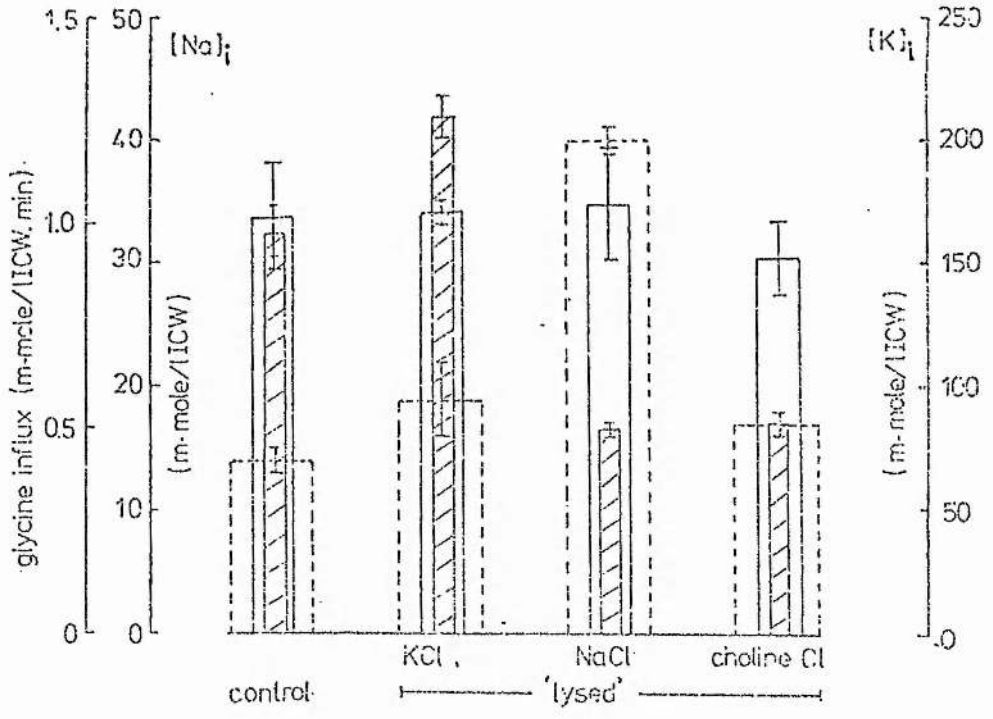


Fig.12.
Effect of intracellular Na and K on glycine influx, Xenopus. Cells 'lysed' for 5min and reconstituted for a further 5min with the chloride indicated. Ion contents measured immediately after treatment. Influx measured from low-K W&Q (2mM glycine*), at room temperature. T-test showed no significant effect of treatments on influx ($P>0.9$ or $P>0.5$), cell number or volume ($P>0.1$). Values are means ± SE of 4 readings for influx and 6 readings for ion contents.
□ glycine influx, [ ] intracellular Na, ▨ intracellular K.

For experiment 2 (Table 10), data were also plotted as S/V : S where V = glycine* influx and S = external concentration of Na. By regression analysis, linear correlations were obtained for each external concentration of glycine (0.5 - 3mM) with $r > 0.96$. This indicated a 1:1 relationship between Na and glycine. The sensitivity of the carrier to Na was independent of the glycine concentration with a Km of external Na of 14mM, (at which Na concentration, half maximal glycine influx would be measured). This could explain the finding that a marked reduction in external Na was necessary before an effect on glycine influx could be detected. For experiment 2, in Table 10, the influx values at 75mM external Na were not significantly different from those measured at 130mM external Na.

Effects of intracellular ion concentrations on glycine uptake

1. Xenopus

'Lysis' Internal ion composition was varied during the 'lysing' procedure by reconstituting cells with either KCl, NaCl or choline Cl. Intracellular Na varied between the control level (14mmole/lICW) and 40mmole/lICW. Intracellular K varied between 83 and 210mmole/lICW. The bar diagram of Fig.12 shows the subsequent glycine influx in control and experimentally treated cells with their associated initial intracellular ion levels. The influxes were not significantly different from the control value (by T-test) despite altered intracellular ion concentrations.

Low $(K)_e$ incubation 1hr incubation at room temperature in W&Q salt solution containing 0.2mM K (compared with the normal 5mM), resulted in a partial block of the sodium pumps. There was a subsequent increase in intracellular Na from 9 ± 0.5 to 25 ± 2mmole/lICW, and a decrease in K from

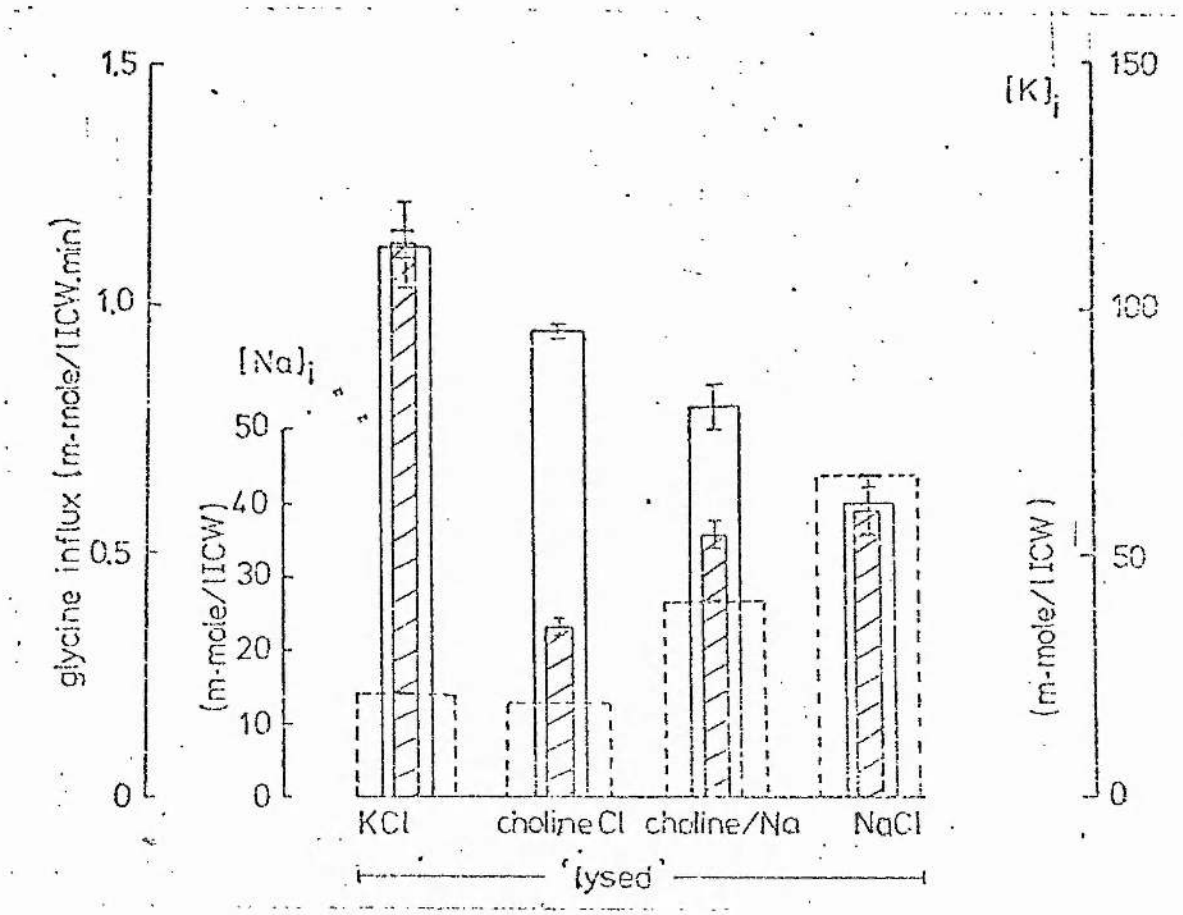


Fig.13.

Effect of intracellular Na and K on glycine influx. 'lysed' HeLa

Cells 'lysed' 5min and reconstituted 5min with the chloride ions shown. Plates rinsed and influx measured in low-K^+ Krebs, glycine* 2mM, over 5min at 37°C. Analysis of variance showed that glycine influx was dependent upon intracellular ion levels, ($P < 0.025$). Ion contents measured immediately after reconstitution of the cells. Values are means of 2 readings ± SE bars. □ = glycine influx, ▯ = intracellular Na, ▨ = intracellular K.

Table 11

Preincubation medium (1hr) (K content, mM)	influx medium (5min)	Vmax (mmole/l ICW.min)	Km (mM)
control (7)	control (7)	10.5	3.19
control (5)	low-K (0.1)	8.3	2.67
low-K (0.2)	control (7)	6.7	1.33
low-K (0.1)	low-K (0.1)	4.0	5.91

Effect of low-K incubation on glycine* influx, HeLa.

Cells preincubated at 37°C in control Krebs or low-K Krebs (KCl omitted) and influx measured over 5min from varying external glycine* concentrations (37°C), in control or low-K Krebs. External Na constant at 140mM. Kinetic constants calculated from S/V : S linear correlations of data. Cell number and volume were not affected by treatment.

135 ± 2 to 111 ± 1mmole/lICW (means ± SE). From the 'lysis' results, this was assumed to be insufficient an alteration to cause any detectable change in glycine* influx.

2. HeLa

'Lysis' Ion contents were altered during 'lysing' treatment. Fig.13 shows glycine* influx measured after 'lysis' and the associated intracellular Na and K levels. All influx values were lower than the control (non-'lysed') value of 1.74 ± 0.06 mmole/lICW. This was assumed to be a non-specific effect due to 'lysis'. There was, however, a further decrease in glycine* influx associated with an increase in intracellular Na and unrelated to the intracellular K variation.

Low-K incubation Because of the possibility that the 'lysis' treatment affected amino acid influx in HeLa cells directly, intracellular ion levels were manipulated by preincubation in low-K Krebs (minus KCl), (for example, in 1 experiment, after 1hr, internal Na was increased from 12 ± 0.9 to 42 ± 2mmole/lICW and internal K concentration decreased from 146 ± 4 to 62 ± 7mmole/lICW). Table 11 shows kinetic constants of influx from 2 similar experiments. In control cells (preincubated 1hr in normal Krebs) removal of extracellular K during the 5min influx period had no significant effect on glycine influx, (by regression analysis of S/V : S plots of the data, and T-tests of slopes and intercepts of the linear correlations obtained).

Incubation in low-K for 1hr caused a decrease in glycine influx, when uptake was measured in low-K Krebs. This was associated with a decreased Vmax ($P < 0.01$), indicating a decreased number or rate of turnover of carriers. The apparent Km increased, though not significantly so.

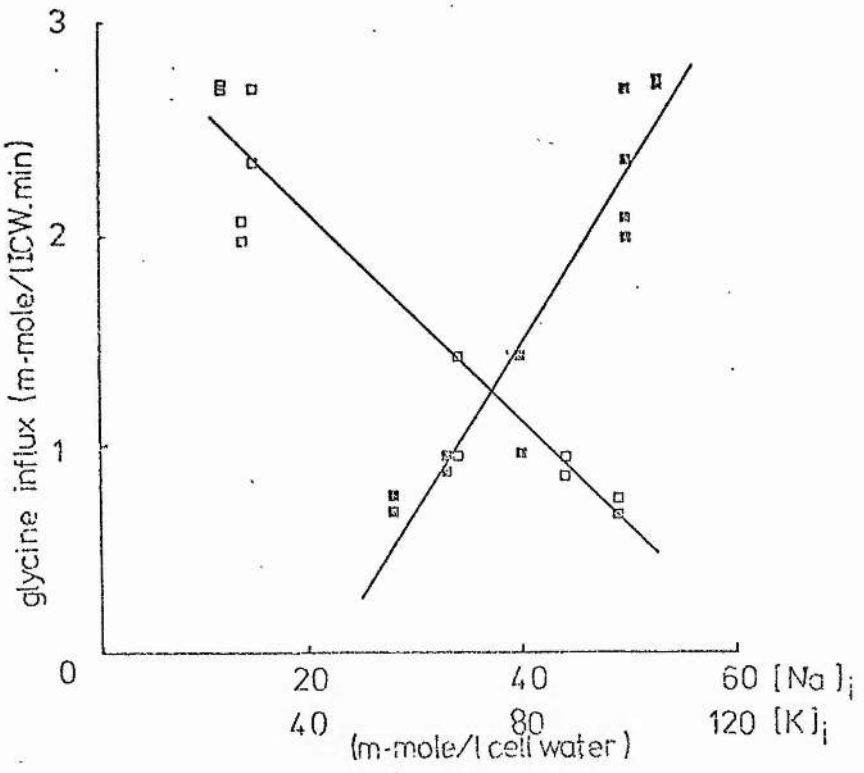


Fig.14.

Effect of intracellular Na and K levels on glycine influx, HeLa.

Cells preincubated in Krebs containing K = 0.1 to 4.7mM; plates rinsed in low-K Krebs and influx measured from Krebs, K = 0.1mM, glycine* 2mM, for 5min at 37°C. Cell number and volume not affected by treatment. Lines drawn by regression analysis. Each point represents an individual reading. Influx correlated with K ■, with Na □.

If influx was measured from normal Krebs, the cells appeared to partially recover from the low-K treatment within the 5min of uptake measurement. This was presumably due to a recovery of normal intracellular ion levels.

In these experiments all the KCl was omitted from the Krebs but by varying the external K during the 1hr preincubation, it was possible to obtain a range of resultant intracellular ion levels. In this treatment, however, unlike the 'lysing' treatment, changes in intracellular Na and K were necessarily linked. Thus, although a gradation in the reduction of glycine influx was observed, it was not possible to distinguish whether the effect was due to an increased internal Na or a decreased internal K. In Fig.14 equally good linear correlations were obtained for glycine influx : intracellular Na ($r = -0.953$) and influx : intracellular K ($r = 0.950$), giving $P < 0.001$.

Low-K incubation with sorbitol replacement of external Na

In an attempt to circumvent this interaction between intracellular Na and K variation, cells were incubated, as previously, with low-K Krebs but, in addition, sorbitol iso-osmotically replaced external Na to a varying extent. With up to 60% substitution of external Na, sorbitol itself had no effect on ion contents ($P > 0.25$) nor on glycine influx ($P > 0.5$). In cells incubated in low-K (0.2mM) the intracellular ion concentrations were altered (K decreased from approx. 120 to 50mmole/lICW and Na increased from approx. 11 to 50mmole/lICW) and glycine influx was reduced, as noted previously. As external Na was replaced with sorbitol, intracellular Na approached a normal value and glycine influx increased from its low level. No correlation was found between glycine influx and intracellular K. There was, however, a linear correlation between influx and intracellular Na ($r = -0.916$), a $v : f(Na)_i$

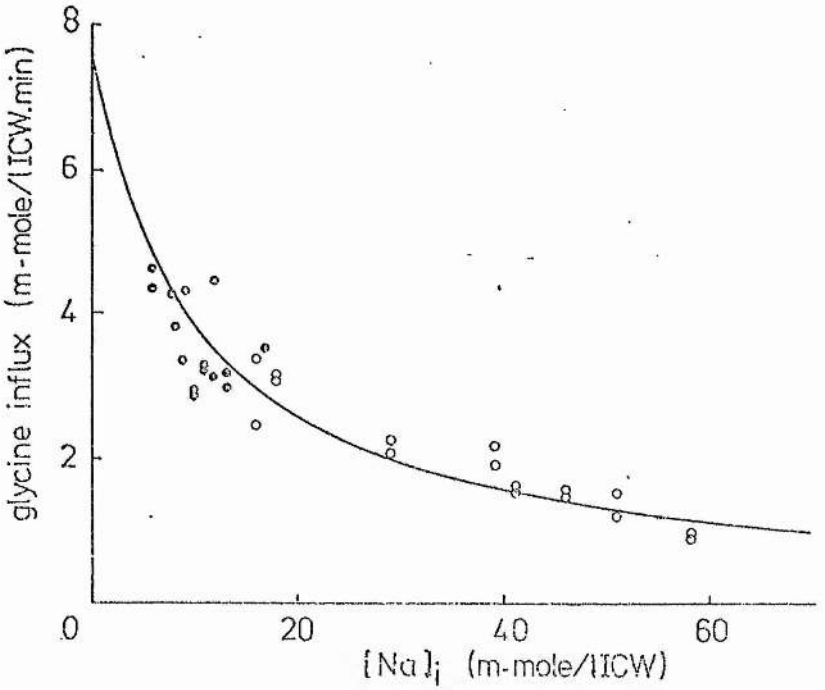


Fig.15.

Effect of intracellular Na^+ on glycine influx, HeLa

Cells preincubated 1hr at 37°C in normal Krebs or low-K Krebs (minus KCl) with Na^+ partially replaced by sorbitol. Glycine influx measured over 5min at 37°C from 2mM glycine* in low-K Krebs, normal Na. No effect of treatment on cell number. By analysis of variance, cell volume was increased up to 14% with sorbitol treatment, ($P < 0.001$). Each point represents an individual reading, normal-K (●), low-K (○). Curve drawn to fit the equation $V = 1/(0.129C + 0.130)$, where V = glycine* influx and C = intracellular concentration of Na^+.

Table 12

reconstituting chloride	intracellular ion concentration (mmole/l ICW)		initial $(\text{glycine})_i$ (mmole/l ICW)	$T_{\frac{1}{2}}$ (min)	rate constant (min^{-1})	efflux (mmole/l ICW.min)
	Na	K				
sodium	23	74	10.5	16.6	0.042	0.438
Na/choline	18	63	7.2	18.0	0.039	0.277
choline	6	52	5.1	18.2	0.038	0.194
Na			9.6	15.2	0.046	0.442
choline			6.0	18.0	0.039	0.232
Na	20	52	6.5	27.6	0.025	0.163
choline	5	43	5.5	22.8	0.030	0.167

Effect of intracellular Na on glycine* efflux from 'lysed' cells. Xenopus.

Cells lysed (10min) and reconstituted (10min) at room temperature. (2-^{3}H) glycine (1.5 to 2.5mM) present extracellularly during treatment. Each efflux rate calculated from 6 fractional loss values per 2 or 3min intervals, from one plate of cells. External medium during efflux was normal W&Q salt solution. Cell number and volume not affected by treatment.

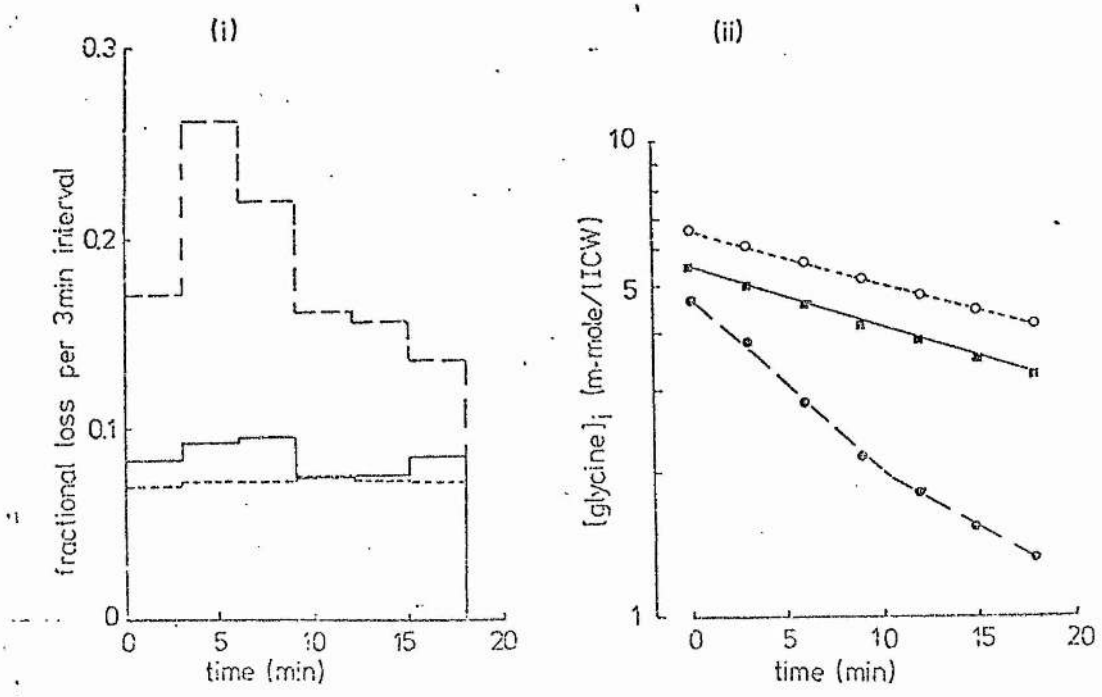


Fig.16 i and ii

Effect of intracellular K on glycine efflux from 'lysed' cells, Xenopus

Cells 'lysed' 10min and reconstituted 10min, in the presence of 1.5mM glycine*. Efflux was into normal W&Q.

(i) expressed as fractional loss. Each histogram from 1 plate of cells, reconstituted with KCl – –, choline Cl ——— , or NaCl - - - - .

(ii) expressed as glycine* content against time. Each point represents an individual reading. Cells reconstituted with Na o , K ● , choline ■. Lines drawn by eye. Each line is from one plate.

relationship. A more acceptable distribution of variance about the linear correlation was found for a $1/v$: $f(Na)_i$ relationship ($r = 0.934$). Fig.15 shows glycine influx plotted against internal Na, with the curve fitted using the slope and intercept values calculated from this latter plot.

Similar results were obtained if Li, instead of sorbitol, was substituted for external Na during low-K incubation.

Effect of intracellular ion concentration on efflux of glycine

Xenopus

Sodium Cells were loaded with (2-^{3}H) glycine and their ionic contents varied during 'lysing' treatment. Efflux of glycine* from cells reconstituted with either Na or choline Cl could be represented by a single exponential, despite the fact that ion contents changed during the total efflux time of 12 to 18min. Table 12 shows the results from 3 experiments. As can be seen, the extent to which the cells could be loaded with glycine* depended on the reconstituting chloride ion. This was probably due to normal glycine accumulation utilising the amino acid transport system during Na reconstitution. Intracellular Na was raised to a level previously shown to be insufficient to cause a reduction in glycine* influx. In these 'high-Na' cells, glycine efflux was increased but this could be simply explained in terms of the increased initial intracellular glycine* concentration.

Potassium Cells were reconstituted by adding KCl or choline Cl to hypotonic 'lysing' solution and glycine* efflux subsequently measured.

Efflux was not a simple exponential but could be analysed into at least two components; an initial fast component, followed by a slower efflux. Fig.16 (i) and (ii) show fractional loss per 3min time intervals and semi-log plots of glycine* content against time from cells 'lysed' and reconstituted with either KCl,

Table 13

reconstituting chloride	intracellular ion concentration (mmole/l ICW)		initial $(glycine)_i$ (mmole/lICW)	$T\frac{1}{2}$ (min)	rate constant (min^{-1})	efflux (mmole/l ICW.min)
	Na	K				
K	7	138	(i) 22.5 (ii) (11.5)	6.1 (11.6)	0.114 (0.060)	2.556 (0.687)
K	5	111	(i) 29.6 (ii) (22.5)	10.9 (20.8)	0.064 (0.033)	1.883 (0.749)
K	7	105	(i) 50.0 (ii) (37.5)	9.3 (14.6)	0.075 (0.048)	3.725 (1.781)
choline	5	46	6.9	22.1	0.032	0.216
choline	8	52	30.5	18.6	0.037	1.138
choline	6	53	75.0	35.0	0.020	1.485
K	8	170	(i) 10.9 (ii) (8.6)	5.4 (7.8)	0.128 (0.089)	1.398 (0.764)
K/choline	4	65	(i) 5.7 (ii) (5.2)	10.5 (12.9)	0.066 (0.054)	0.376 (0.279)
choline	4	43	5.3	13.0	0.053	0.282
K	12	203	(i) 8.6 (ii) (5.8)	8.2 (16.0)	0.085 (0.043)	0.727 (0.251)
K/choline	10	182	(i) 8.1 (ii) (6.9)	10.1 (16.2)	0.069 (0.043)	0.557 (0.295)
K/choline	13	147	(i) 7.5 (ii) (6.8)	12.4 (16.1)	0.056 (0.043)	0.419 (0.292)
K	6	123	(i) 4.8 (ii) (3.4)	8.1 (13.2)	0.086 (0.053)	0.411 (0.176)
choline	5	43	5.5	22.8	0.030	0.167
K	3	157	7.4	7.0	0.099	0.733
choline	6	60	3.4	15.4	0.045	0.151
K			(i) 6.1 (ii) (4.3)	7.6 (16.2)	0.091 (0.043)	0.556 (0.184)
choline			6.5	22.4	0.031	0.201

Effect of intracellular K on glycine* efflux from 'lysed' Xenopus cells.

Table 13 (Continued)

Cells 'lysed' 10min and reconstituted 10min with chloride ion shown. (2-^{3}H) glycine present during treatment, to give initial intracellular glycine* levels indicated. Brackets indicate values calculated from the second component of efflux, when present. Cell number and volume not affected by treatment.

NaCl or choline Cl in one experiment. Efflux was increased in cells with a high internal K but within 6 to 8min recovered to a value more comparable with that of Na or choline reconstituted cells. Within 6min the ion contents of refilled cells approached normal values.

Table 13 gives the results from several experiments. The bracketed values were calculated from the second, slower component of efflux from 'high-K recovered' cells. Direct correlation of efflux with internal K, for all data, was not possible because of variations in initial intracellular glycine* which, itself, altered the efflux rate; nor was it possible to correlate glycine efflux with initial intracellular glycine, because of variations in intracellular K.

It was, however, apparent that as intracellular K was increased, the efflux rate of glycine* increased. Although reconstituting with K tended to produce an increased initial intracellular glycine*, this was not always the case; the increased initial efflux rate could not be explained in terms of this additional intracellular glycine*.

Insufficient data was collected for separate kinetic analyses of glycine* efflux from 'high-K recovered' and Na reconstituted cells. When values were added to efflux values from choline reconstituted cells, however, the linear correlation of s/v : s was not significantly different from that obtained by analysing the choline data alone (slope, $P > 0.9$, intercept, $P > 0.5$) and the variances about the lines were not significantly different ($P > 0.25$). For choline reconstituted cells, Vmax = 3.85mmole/lICW.min, Km = 85.0mmole/lICW; for choline + others, Vmax = 4.00 mmole/lICW.min, Km = 84.0mmole/lICW.

Summary It appeared, therefore, that Xenopus cells reconstituted with either Na or choline had efflux rates which were similar and did not vary with intracellular ion contents, (neither Na nor K).

Table 14

30 min pretreatment	treatment	ATP (mM)	glycine influx (mmole/lICW.min)
W&Q	control		0.71 ± 0.05 (5)
	lysis	nil	0.73 ± 0.03 (4)
		1	0.69 ± 0.02 (5)
		2	0.71 ± 0.04 (2)
		4	0.68 ± 0.01 (2)
		6	0.61 ± 0.04 (2)
DNP + IAA	control		0.70 ± 0.01 (2)
	lysis	nil	0.60 ± 0.05 (7)
		1	0.66 ± 0.07 (5)
		2	0.73 ± 0.01 (2)
		4	0.58 ± 0.05 (2)
		6	0.70 ± 0.05 (2)

Effect of DNP + IAA on glycine influx into normal and lysed Xenopus cells.

Cells incubated at 27°C in W&Q salt solution +/- 2,4 - di nitrophenol (DNP), 10^{-3}M, and iodo-acetic acid (IAA), 10^{-4}M. Plates then rinsed and cells incubated for a further 10min in W&Q or lysed (5min) and reconstituted by addition of KCl (5min). ATP present in the lysing solution at the concentrations shown. Glycine influx measured over 5min from 2mM glycine*. Analysis of variance or T-test showed no significant effect of treatment on cell volume. DNP + IAA incubation results in a significant loss of cells from the plates (generally about 10% of the control). Data are from 3 experiments, the number of readings shown in brackets. Mean ± SE given.

Table 15

30 min pretreatment	treatment	ATP (mM)	glycine influx (mmole/l ICW.min)
W&Q	lysis	2	0.87 ± 0.02 (3)
DNP + iodo-acetamide	lysis	nil	0.89 ± 0.04 (3)
		2	0.87 ± 0.06 (3)
		4	0.96 ± 0.15 (3)

Effect of DNP + iodoacetamide on glycine influx into 'lysed' Xenopus cells.

Cells incubated at 27°C in W&Q salt solution +/- DNP (10^{-3}M) + iodoacetamide (2 x 10^{-3}M). Plates rinsed and cells 'lysed' (5min) and reconstituted with KCl (5min). ATP present in the lysing solution at the concentrations shown. Glycine influx measured over 5min from 2mM glycine*. DNP/iodoacetamide caused a significant loss of cells (about 40% of the control) but ATP had no effect ($p > 0.75$). Cell volume was unaffected ($p > 0.4$). Number of observations shown in brackets. Mean ± SE given.

When cells were reconstituted with K, intracellular K was increased above the control level and fell to normal within 6min. This was associated with an increased glycine* efflux which also recovered with about the same time course. The discrepancy between measured initial efflux rates and those estimated using the calculated kinetic parameters was not constant and was not related to intracellular K in a simple manner.

Glycine uptake after treatment with metabolic inhibitors

Xenopus cells Table 14 shows the effect of pre-equilibration in DNP (10^{-3}M) and IAA (10^{-4}M) on the subsequent glycine* influx into normal and 'lysed' cells. T-test of the results showed that there was no significant effect of either metabolic inhibitor treatment ($P > 0.9$), or 'lysis' either in control cells, ($P > 0.5$), or in DNP + IAA treated cells, ($P > 0.2$). ATP present during 'lysis' had no significant effect at any of the concentrations used.

It was possible that iodo-acetic acid injured the membrane in some way, indirectly causing loss of cells, so similar experiments were carried out, replacing IAA with iodoacetamide (2×10^{-3}M). The results are shown in Table 15. T-test showed no significant effect of DNP/iodoacetamide on glycine uptake, ($P > 0.9$). Analysis of variance showed no significant effect of ATP present during 'lysis', after DNP treatment, ($P > 0.75$).

This occurred despite an altered internal ionic composition. 1 hour incubation with DNP + IAA resulted in an increased internal Na concentration (control = 9 ± 2, DNP/IAA = 17 ± 3, $P < 0.05$) and a decreased internal K concentration, (control = 148 ± 5, DNP/IAA = 97 ± 9, $P < 0.001$).

Cells 'lysed' and refilled with Na are able to recover a normal internal Na when placed into a high Na medium, independent of any DNP/IAA treatment and the concentration

Table 16

Growth	experimental medium	Vmax mmole/lICW.min	Km (mM)
control	control (W&Q)	1.54	3.18
control	ouabain (10^{-3})	1.64	4.43
control	control	0.90	2.71
ouabain$_8$ ($5x10^{-8}$M)	control	0.99	2.56

Effect of ouabain on glycine influx. Xenopus.

After growth, plates rinsed in W&Q salt solution and glycine influx measured over 5min (at room temperature) from varying concentrations of external glycine*. Straight line correlations of the data were obtained using Eadie plots (regression analyses $P < 0.001$). Ouabain affected neither cell numbers per plate nor cell volume. (T-test, $P > 0.05$).

of ATP, so that it was possible that the cells recovered from metabolic poisoning within the time of the experiment. (The results of Lamb and Lindsay (1971), suggest that this is unlikely. In L-cells, ATP incorporation was necessary for active Ca efflux to occur after DNP/IAA poisoning). To check this, in normal (non-lysed) cells, plates were pre-equilibrated for ½h in DNP (10^{-3}M) and IAA (10^{-4}M) and the uptake subsequently measured in the presence of the inhibitors. In Xenopus cells, the 10min uptake was not different from that into control cells, pre-equilibrated in W&Q salt solution. In HeLa cells, neither 5 nor 15min glycine* contents were significantly different from the control values, (by analysis of variance, $P > 0.1$).

Effect of ouabain on glycine uptake

1. Xenopus

   External ouabain on glycine influx

   Kinetic analysis of influx of glycine into Xenopus cells measured in control conditions, or in the presence of ouabain (10^{-3}M) without pretreatment, or after 24hr growth in the presence of ouabain (5×10^{-8}M), are shown in Table 16. There was no significant effect of ouabain treatment on either Vmax or the apparent Km of influx. Xenopus cells have a low sensitivity to ouabain with respect to sodium pump activity. This is supported by the fact that after 24hr growth in medium containing 5×10^{-8}M ouabain, internal Na and K concentrations were not significantly different from control levels (Na = 14, K = 86 mmole/l ICW). If ouabain is considered to act indirectly via a block of the Na-K-pump, then this and the insensitivity of glycine influx to intracellular ion levels in Xenopus cells could be offered as an explanation of the lack of effect.

Table 17

treatment	ouabain concentration (M)	glycine influx (mmole/lICW.min)
control	nil	0.94 ± 0.03 (2)
'lysed'	nil	1.04 ± 0.06 (2)
	10^{-5}	1.28 ± 0.15 (2)
	10^{-4}	1.01 ± 0.01 (2)
	10^{-3}	1.08 ± 0.03 (2)

Effect of 'lysis' in the presence of ouabain on glycine influx in Xenopus cells.

Cells incubated at 27°C for 10min in W&Q salt solution or 'lysed', ie. 5min lysis + 5min reconstitution with KCl. Ouabain was present in the lysing solution at concentrations indicated. Glycine influx was measured over 5min from 2mM glycine* in W&Q salt solution. Number of observations given in brackets. Mean ± SE shown. There was no significant effect of ouabain on cell volume or number and no effect on internal Na or K concentrations, measured immediately after 'lysis' was complete.

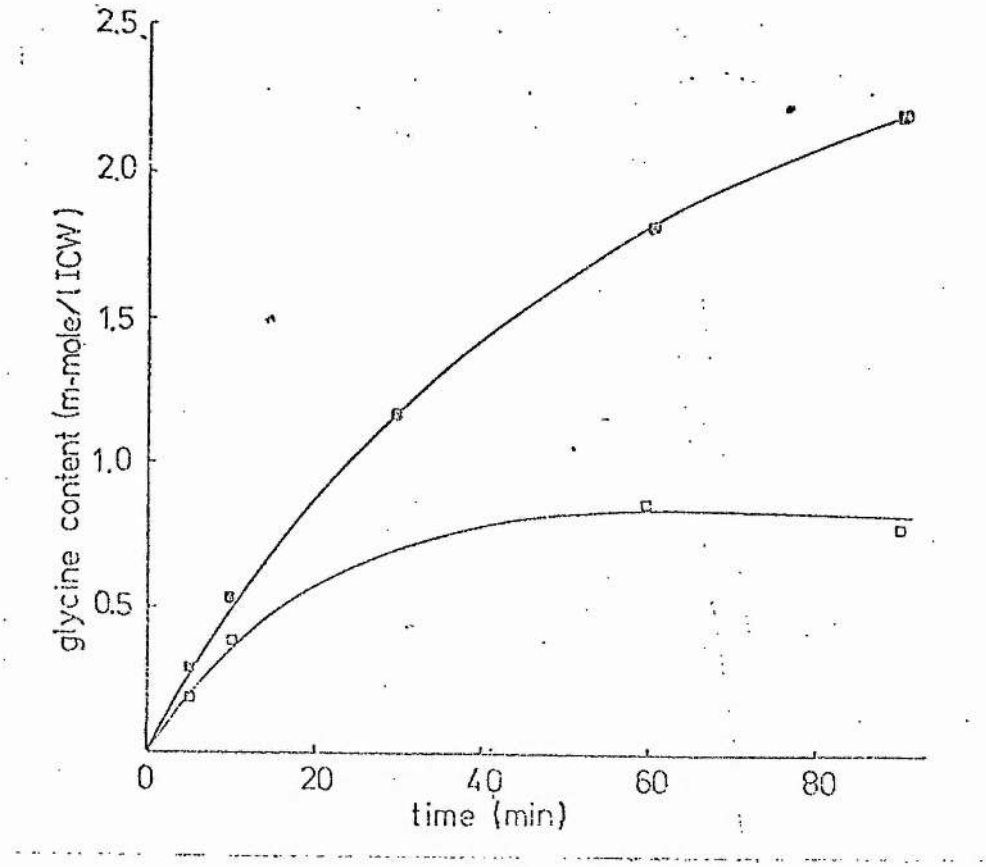


Fig.17.

Effect of ouabain on glycine accumulation in Xenopus cells

Cells equilibrated 30min in W&Q salt solution and then incubated at room temperature in W&Q +/- ouabain (10^{-3}M) containing 0.1mM glycine*, for the times shown on the abscissa. There was no effect on cell number or volume. Points are individual readings. Lines drawn by eye. control, ■ ; with ouabain, □ .

Table 18

Growth	experimental medium	Vmax (mmole/l ICW.min)	Km (mM)
control	control (Krebs)	15.38	1.58
control	ouabain (10^{-3}M)	14.27	1.72
control	control	9.17	2.58
ouabain (5×10^{-8}M)	control	4.25	2.15

Effect of ouabain on glycine influx. HeLa.

After growth, plates rinsed in Krebs and glycine* influx measured over 5min (at 37°C) from varying external glycine concentrations. Vmax and Km calculated from the data using Eadie plots. In all correlations, $P < 0.001$. There was no significant effect of ouabain on either cell volume or number during acute treatment, or cell volume in the growth experiment. 24hr growth in ouabain resulted in a significant loss of cells ($P < 0.001$), to 88% of the control. Neither Vmax or the apparent Km were significantly altered by acute treatment with ouabain. Growth in ouabain resulted in a decreased Vmax ($P < 0.001$) with no significant change in the apparent Km of influx.

Internal ouabain on glycine influx

Table 17 shows that 'lysis' had no significant effect on glycine influx measured immediately after reconstitution was complete, (T-test, $P>0.2$). Analysis of variance showed that there was no significant effect of ouabain present during 'lysis', ($P>0.1$). Although, at present, there is no direct evidence that ouabain (MW = 728) enters the cell during 'lysis', studies of the binding of radioactively labelled ouabain during 'lysis' suggest that there is labelling additional to that bound to the outer membrane of the cell. (With an external ouabain concentration of 10^{-3}M, the additional uptake is approximately 2×10^{-4} mmole/l ICW). If this represents internal ouabain, then the results suggest that there is no direct effect of internal ouabain on the glycine influx into Xenopus cells.

External ouabain on glycine accumulation

Although the influx was not immediately affected by the presence of ouabain, Fig. 17 shows that the capacity of the cells to accumulate the amino acid became less. After 90min incubation with 10^{-3}M ouabain, the glycine* content was only 38% of the control value.

A similar result was obtained in an additional experiment. 30min incubation with 10^{-3}M ouabain resulted in a glycine* content of 1.10, compared with a control value of 1.60 mmole/l ICW. This was associated with an increase in internal Na^+ (control = 9, ouabain treated = 22) and a decrease in internal K^+ (control = 143, ouabain treated = 122, where contents are expressed as mmole/l ICW.)

2. HeLa

External ouabain on glycine influx

Table 18 shows that there was no immediate effect of 10^{-3}M ouabain on the kinetic parameters of glycine influx into HeLa cells.

When cells were grown in B.M.E. containing ouabain (at a lower concentration of 5×10^{-8}M, to reduce cell death) there was, however, a significantly reduced 5min glycine* influx. This was characterised by a reduced Vmax with no change in the apparent Km. There was an associated increased internal Na^+ concentration (control = 21, ouabain treated = 78mmole/lICW) and a reduction in internal K^+, (control = 128, ouabain treated = 76mmole/l ICW).

This reduction in transport was also apparent when influx (from varying external glycine concentrations) was measured in Krebs with a Na content of 1.6mM, compared with the control of 127mM. (Choline replaced Na in an iso-molar manner). Linear correlations of influx : concentration were obtained and the slopes calculated as 0.27 for control and 0.10 for ouabain-treated cells. If uptake at low external Na is still carrier mediated, then this would indicate a change in carrier activity rather than an increased flux due to a changed Na influx.

External ouabain on glycine accumulation

Ouabain, although having no direct effect on influx, caused a decrease in the ability of HeLa cells to accumulate glycine*. (A result previously found in Xenopus cells). After 60min incubation in Krebs containing 10^{-4}M glycine*, intracellular glycine content (in one experiment) was found to be 0.91mmole/l ICW in control cells but only 0.54mmole/l ICW in cells incubated in the presence of 10^{-5}M ouabain.

'Conditioning' effect

When growth medium was changed to 'fresh' medium, there was a 40-50% reduction in experimentally measured glycine* influx in both HeLa and Xenopus cells. This could be avoided by using medium which

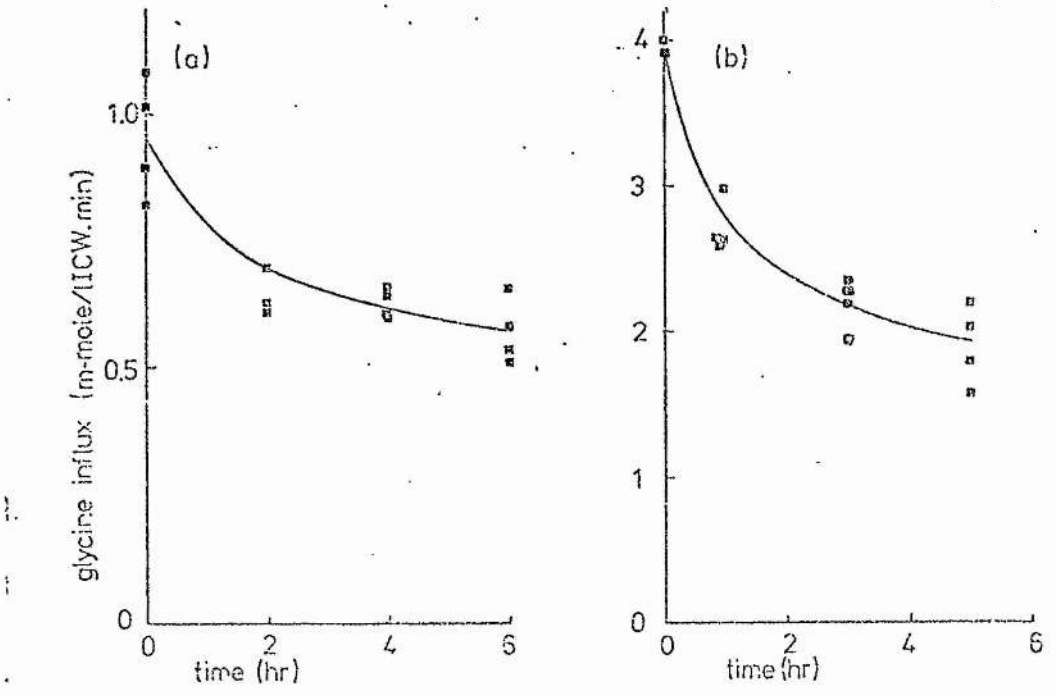


Fig.18.

Effect of 'fresh' medium change on subsequent glycine influx, (a) Xenopus, (b) HeLa

Cells grown normally for 3 days. At time zero, medium changed to fresh W&Q or B.M.E. Glycine* influx measured at the times shown on the abscissa from 2mM external glycine*, in W&Q salt solution, or Krebs, (5min at 26 or 37°C). Values are single observations. Lines drawn by eye.

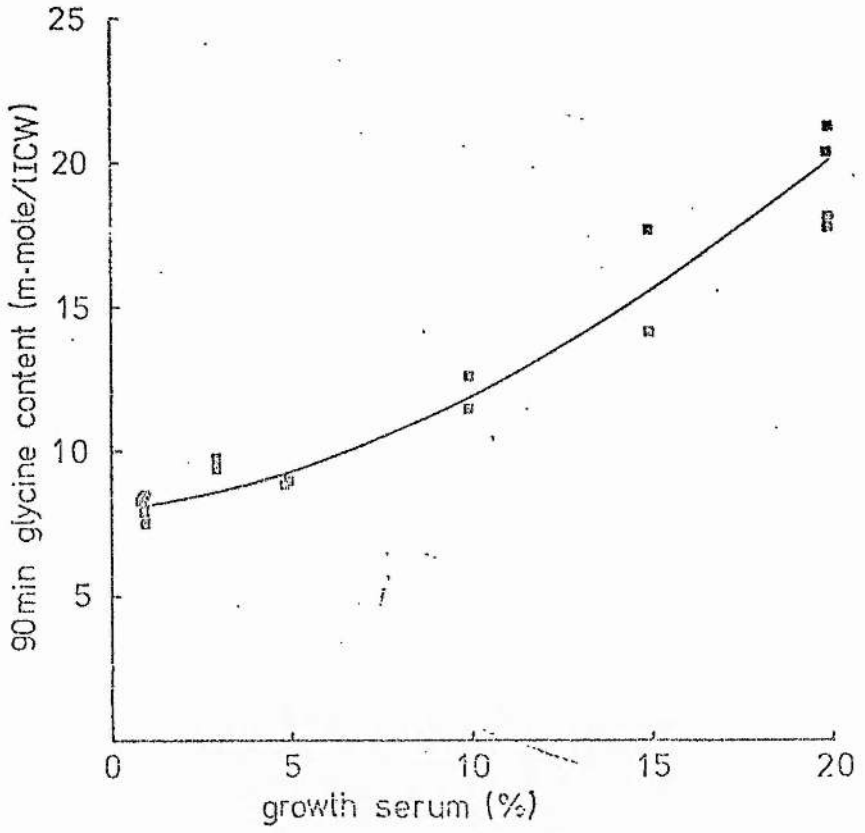


Fig.19.

Effect of growth in various serum concentrations on subsequent glycine transport, Xenopus

Cells grown in medium supplemented with foetal bovine serum at the concentrations shown on the abscissa. Glycine content was measured after 90min incubation at room temperature in W&Q salt solution (1% serum) + 2mM glycine*. Analysis of variance showed a significant increase in the ability of the cells to accumulate glycine as the concentration of serum in their growth medium was increased, ($P < 0.001$). Similar results were obtained in 1 further experiment. Cell number was significantly greater in high-serum grown cells (up to 17%). Cell volume varied with serum concentration; was greatest at 5-10%, with about equal volume in 1 and 20% serum (SE was ± 3% of the mean volume). There was no consistently significant effect on intracellular Na or K. Each point is an individual reading. Line drawn by eye.

had previously supported cell growth; ie. 'conditioned' medium.

The 'conditioning effect' occurred in both normal and 'lysed' cells, grown in normal and low-glutamine medium. It appeared that the reduction was independent of the composition of 'fresh' medium in that there was no immediate effect of low-Na, low amino acid concentration, variation in serum concentration, (from 1 to 20%), or addition of cycloheximide.

Typical results from Xenopus and HeLa are shown in Figs.18 a, b. The mean half-time of loss of uptake activity was 5.4 $\pm$ 0.5 hr (from 4 experiments, 2 with each cell type). Activity was recovered by 36hr.

Kinetic analysis of influx, 2hr after growth medium was replaced by 'fresh' BME, showed that, in HeLa, the decreased influx was associated with a reduced Vmax, ($P < 0.001$), from 20.8 to 9.4 mmole/l ICW.min. There was no change in the apparent Km; control = 0.88mM, after medium change = 0.98mM. (Results calculated from linear correlations of s/v : s plots of influx (v) and external glycine* concentration (s)).

Xenopus cells observed during a medium change, using Nomarski interference microscopy, showed no apparent alteration in shape or size, up to 6min after the change.

Serum effects on glycine uptake

Xenopus

Preliminary experiments showed that when cells were grown for 4 days in medium containing high concentrations of serum, they had a greater ability to transport glycine. This was apparent in both influx and accumulation measurements and was roughly proportional to the concentration of serum. Fig 19 shows accumulation values. Uptake was carried out in 1% serum in order to minimise the competitory effects of serum amino acids, (Kuchler & Marlowe-Kuchler, 1965).

Table 19

Experiment number	% serum during growth	Vmax (mmole/l ICW.min)	Km (mM)
1	0.5	0.47	2.91
	10	0.74	2.86
	20	1.03	2.03
2	1	0.63	2.20
	20	1.20	2.28
3	1	0.36	3.66
	20	1.47	3.33

Effect of serum concentration during growth on kinetic constants of glycine influx.

Cells exposed for 5min (at room temperature) to various glycine concentrations. Data gave linear correlations ($P < 0.001$) for s/v : s plots. Cell number per plate was increased at higher serum concentrations and volume varied as before.

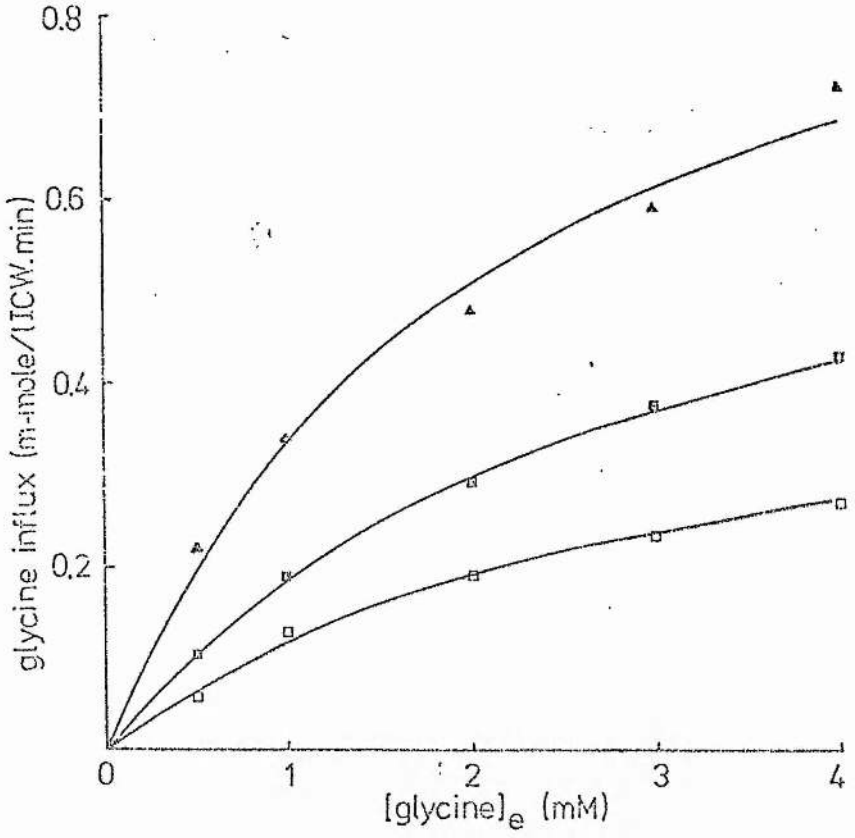


Fig.20.

Stimulation of glycine influx by growth of cells in high serum concentrations. Data from 5min fluxes at external concentrations of glycine* shown on the abscissa, and analysis given in Table (expt. 1). Cells grown for 4 days in media supplemented with 0.5% (□), 10% (▣) or 20% (▲) foetal bovine serum. Points represent single observations. Lines drawn to fit Michaelis-Menten equation, using calculated kinetic constants.

Increased influx was still significant when 5min uptakes were measured in low-Na media, ($P < 0.001$). This indicated that the effect was not simply due to an increase in Na permeability but was a result of some modification of glycine carrier activity.

Table 19 shows the results of kinetic analyses of glycine* influx into cells grown in various serum concentrations. Data were analysed using s/v : s plots, in which the slopes (1/Vmax) were significantly different ($P < 0.01$ or $P < 0.001$). Using v : v/s plots, the intercepts (Vmax) were significantly different but in no experiment was Km (-slope) significantly altered, ($P > 0.5$ or $P > 0.2$). This suggested an increase in carrier number or mobility without an alteration in the affinity of the carrier for amino acid. Fig. 20 illustrates the effect seen in Table 19.

As evidence exists that growing cells produce inhibitors of transport whose effects are counteracted by serum, (see introduction), it was of interest to study the time course of development of the observed serum dependance. If glycine uptake was measured from W&Q medium, instead of W&Q salt solution, there was no immediate effect of medium serum concentration either on influx or 90min content, ($P > 0.5$, by analysis of variance). In this instance the competing amino acids in the medium were used to swamp the serum amino acid effect. The appearance of the effect was slow. In 3 out of 4 experiments, there was no significant difference in cells grown for 24hr in 1 to 20% serum. The stimulatory effect of serum was apparent after 48hr and was fully developed in 4 days. Cells grown for 4 days in 1 or 20% serum retained their different transport properties 24hr after the medium was changed back to 10%. Both appearance and disappearance times were therefore longer than the cell doubling time of 22hr.

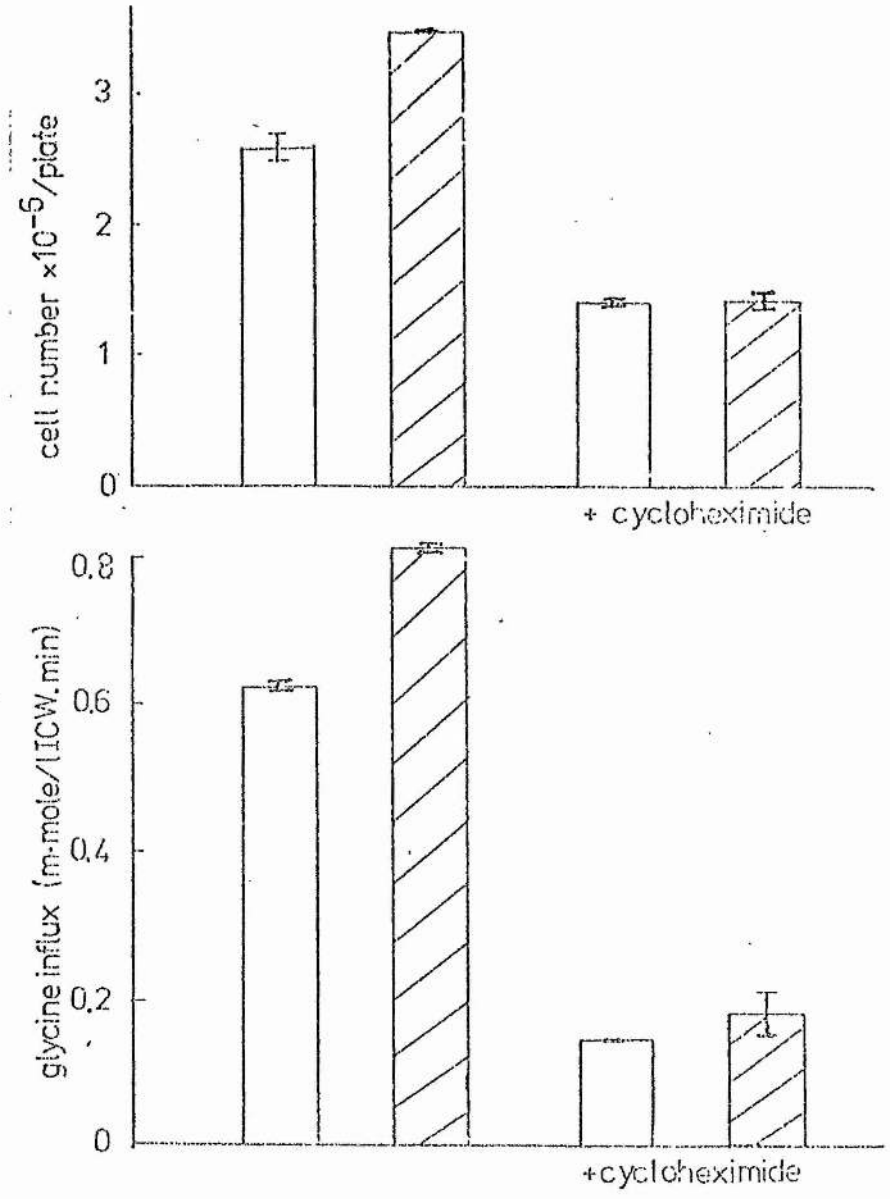


Fig.21.

Cycloheximide interaction with the serum stimulatory effect

Cells grown for 3 days in medium + 10% serum, and for 48hr in either 1 (open bars) or 20% serum (hatched bars) +/- cycloheximide (4 μg/ml). Glycine influx measured over 5min from 2mM glycine*. Growth in 20% serum stimulated cell division. ($P < 0.02$). Cycloheximide prevented cell division, and caused some cell death, (floating cells). Cell volume was reduced by cycloheximide, (by 10% in all serum conditions). Glycine influx was significantly increased in high serum cells, ($P < 0.01$), and markedly reduced by cycloheximide treatment. Values are means of 2 readings ± SE bars.

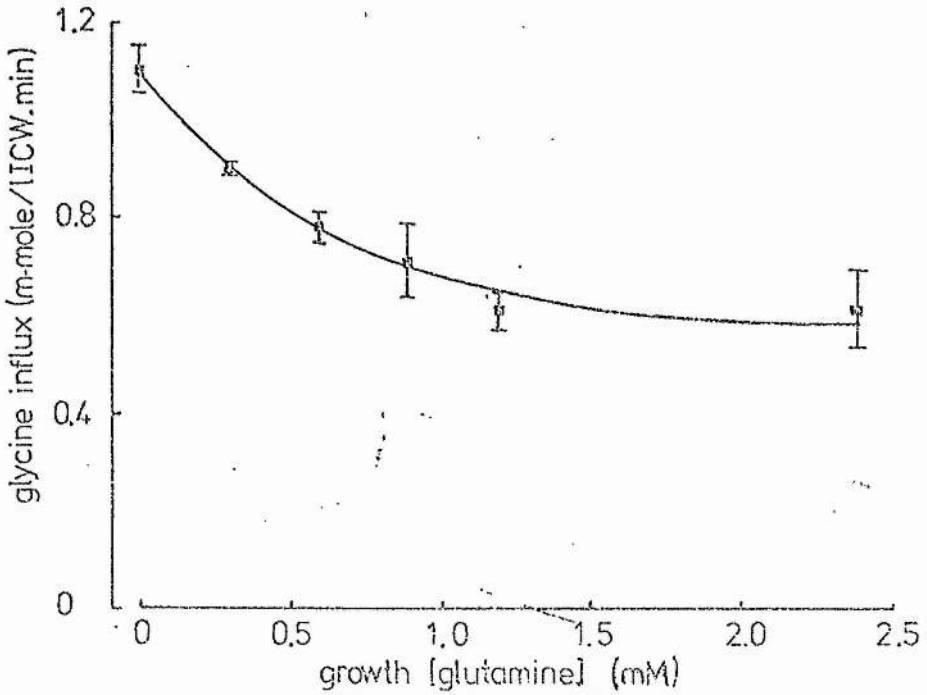


Fig.22.

Growth in varying amino acid concentrations on glycine* influx, Xenopus

Cells grown for 3 days in W&Q medium containing the concentrations of glutamine shown on the abscissa. Glycine* influx subsequently measured over 5min (at 26°C) from 2mM glycine* in W&Q salt solution. Cell volume not affected by treatment. Points are means of 3 readings ± S.E. bars. Line drawn by eye.

(At this stage, time course studies were complicated by a superimposed loss of transport activity when growth medium was changed to fresh medium; see 'conditioning effect').

Fig. 21 shows the effect of adding cycloheximide to growth medium on cell number and glycine influx. The results show that protein synthesis was necessary for cell survival, for maintenance of normal glycine transport function and for the stimulatory effect of serum to occur; that is, for the acquisition of additional transport capacity.

HeLa

HeLa cells were able, after growth in media containing varying concentrations of calf serum, to modify their glycine uptake system in a manner qualitatively similar to that found in Xenopus cells. Detailed experiments were not carried out.

Effect of growth in varying amino acid concentrations

The concentration of amino acids in W&Q growth medium is about 3mM (see Table 2). Of this, A-mediated amino acids make up 2mM, of which 1.2mM is glutamine. In most experiments, the external amino acid concentration during growth was varied by simply adding different concentrations of glutamine. Fig. 22 shows that a decrease in external glutamine during growth resulted in an increase in the uptake capacity of Xenopus cells (by analysis of variance, $P < 0.001$). Conversely, the presence of glutamine during growth suppressed (or repressed) the experimental uptake of glycine. Doubling the normal growth concentration, however, had no further suppressive effect. HeLa cells showed qualitatively similar results, (Hume & Lamb 1974). Glycine influx was reduced equally by growth in glutamine or a mixture of the essential amino acids.

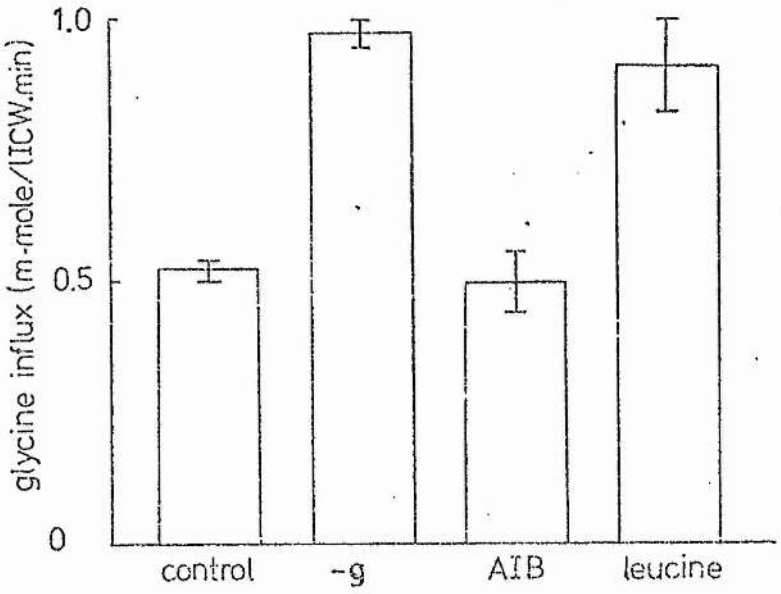


Fig.23.

Action of A and L-mediated amino acids on the glutamine effect, Xenopus

Cells grown for 4 days in normal medium (1.2mM glutamine), medium minus glutamine, - glutamine/+ AIB, - glutamine/+ L-leucine. Glycine influx measured over 5min from 2mM glycine*, (26°C). Cell volume 2% smaller in '-glutamine grown' cells. Values are means of 3 readings ± SE bars.

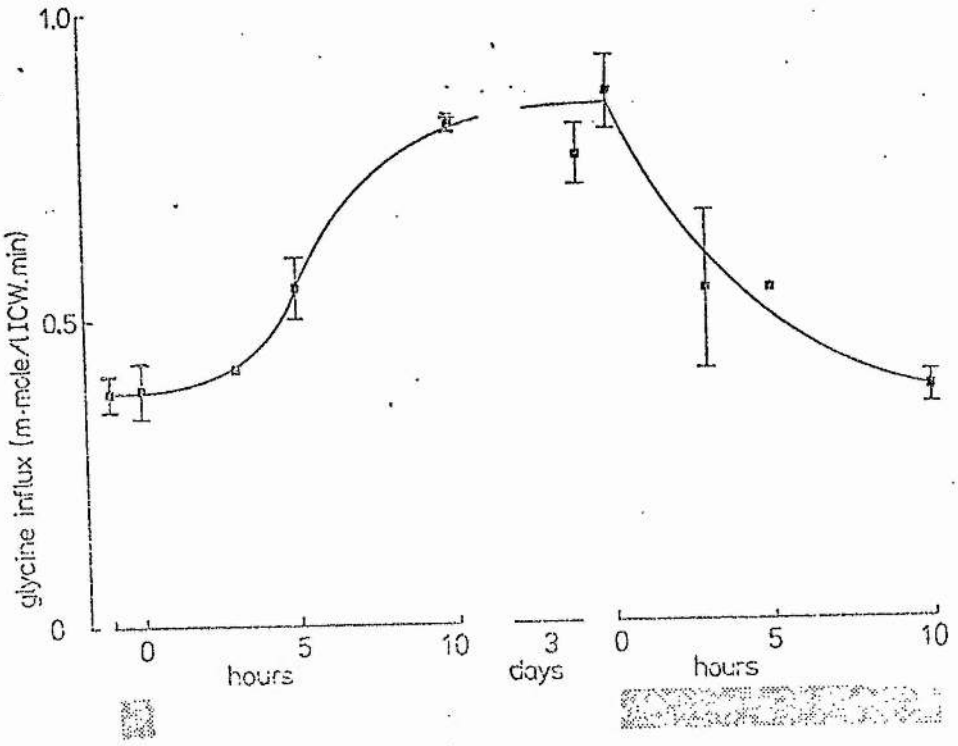


Fig.24.

Time course of additional glycine uptake capacity. Xenopus

Cells grown for 3 days +/- glutamine and growth-medium collected and redistributed at time zero. Base lines of normal and low glutamine cells did not alter significantly over the 10hr (by analysis of variance, $P > 0.1$, $P > 0.75$, respectively). Shaded area along abscissa indicates normal medium. Points are means of 2 readings $\pm$ SE bars. Lines drawn by eye.

To test whether the suppressive effect of growth amino acid concentration on amino acid uptake was confined to those amino acids which were transported by the same system as the experimental amino acid (glycine), glutamine in W&Q was replaced by other amino acids. Fig. 23 shows the results of isomolar replacement of glutamine with the A-mediated amino acid analogue AIB, and the L-mediated amino acid, L-leucine. AIB was able to mimic glutamine, so that glycine influx was not significantly different in cells grown in medium with 1.2mM glutamine or 1.2mM AIB, (by T-test, $P > 0.5$). This result was found in 2 further experiments with Xenopus and in 1 experiment using HeLa cells. Influx values in 'low-glutamine' and 'low-glutamine/+ leucine' cells were increased above that in control cells ($P < 0.001$, $P < 0.05$, respectively) and were not significantly different, ($P > 0.5$). Thus A-mediated amino acid uptake could be suppressed by the presence of A-mediated amino acids during growth but not by L-mediated.

The time course of the modification of transport was studied. When a change of medium was necessary, medium which had previously supported cell growth was used in preference to fresh medium in order to avoid the 'conditioning effect'. Fig. 24 shows the time courses of development and loss of additional uptake activity in Xenopus cells. Increased influx occurred after a variable time lag to a plateau at about 10hr. There was no further increase up to 4 days. Increased uptake capacity decreased with a half-time of about 3½hr, when cells were returned from a low-glutamine to normal medium until, at 10hr the uptake was again at the control level. In a further experiment the half-time of decay of the additional uptake capacity was 2 hr.

The results suggested a modification of the transport carrier system. Kinetic analyses of influx into Xenopus cells showed that growth minus glutamine

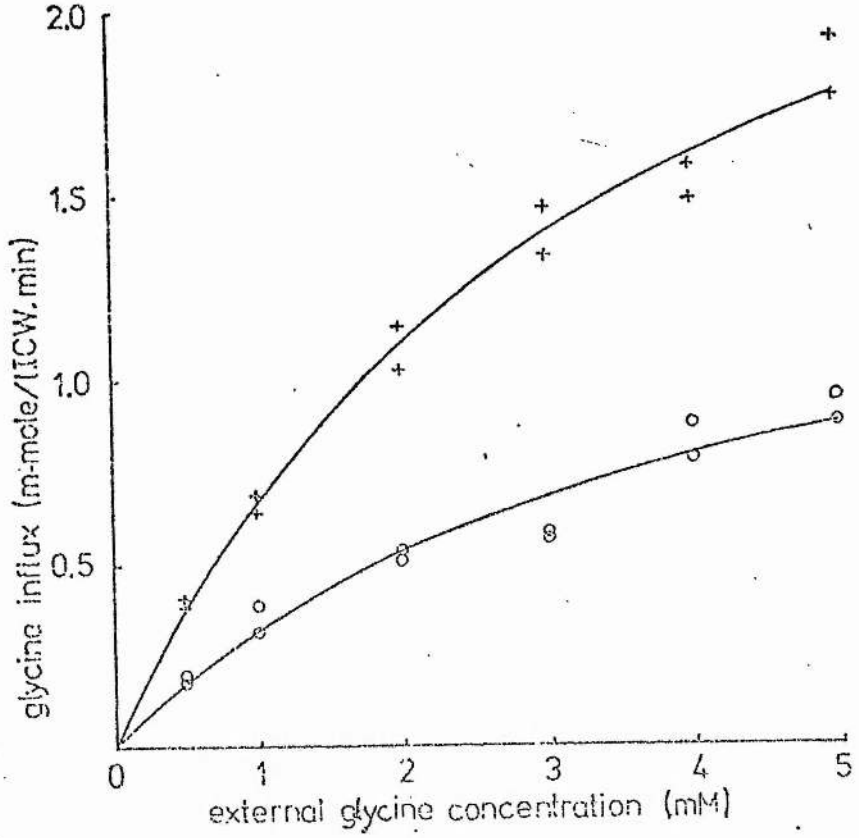


Fig.25.

Glycine influx kinetics in normal and low-glutamine grown cells. (Xenopus)

Cells grown 4 days in normal W&Q medium (o), of medium minus glutamine, (+). Glycine* influx measured over 5min (26°C) from varying external glycine concentrations. Each point represents an individual reading. Lines drawn to fit the Michaelis-Menten equation, with kinetic constants calculated from s/v : s plots of the data (linear correlations of $P < 0.001$). Vmax, but not Km values, significantly different. No effect of treatment on cell number. Cell volume decreased by 11% in -glutamine cells, ($P < 0.001$).

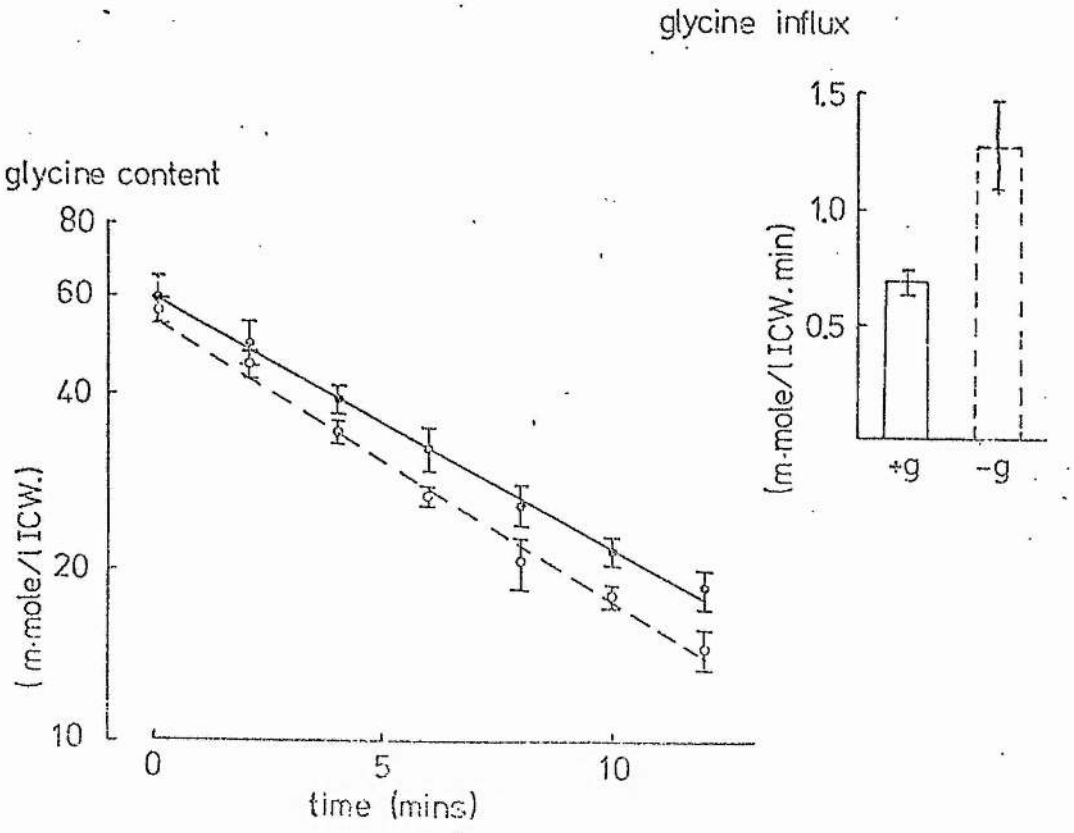


Fig.26.

Glycine efflux from 'lysed' and refilled cells, grown normally and minus glutamine, Xenopus

Cells grown 3 days in normal (●) or minus glutamine (○) medium. Cells 'lysed' and reconstituted in the presence of 15mM glycine*. During efflux, radioactive effluents were collected per 2min intervals and residual glycine* contents calculated. Influx measured in the same group of cells, from 2mM glycine*. No effect of growth or experimental treatment on cell number. Cell volume decreased by 9% in -glutamine cells. Points are means of 3 readings, $\pm$ SE. Lines drawn by regression analysis (with correlations $P \leq 0.001$). T-test of slopes and intercepts not significantly different.

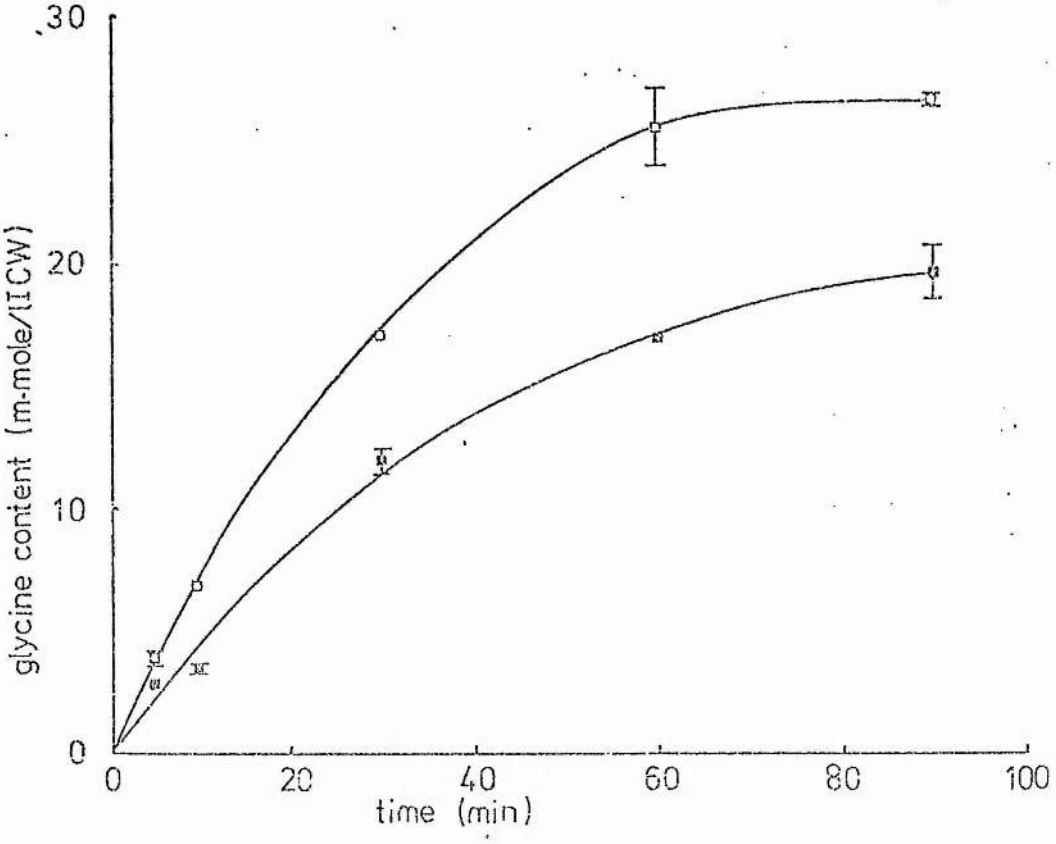


Fig.27.

Glycine accumulation in control and low-glutamine cells, Xenopus

Cells grown 3 days in normal (▣) and -glutamine (□) media. Glycine uptake measured from 2mM glycine* (at 26°C) in W&Q salt solution. Influxes were; control = 0.53 ± 0.01, -glutamine = 0.80 ± 0.06 mmole/lICW.min. Analysis of variance showed a significant interaction of +/- glutamine effect with time, ($P < 0.005$). Points are means of 2 readings ± SE bars. Lines drawn by eye.

caused a significant increase in Vmax from 1.52 mmole/1ICW.min in cells grown in normal glutamine medium to 2.97 mmole/1ICW.min in cells grown minus glutamine, (see Fig. 25). Km was not significantly altered ($p > 0.5$, from slopes of v : v/s plots), = 3.70mM in control cells and 3.42mM in low-glutamine cells. HeLa cells gave more complex results. In 2 experiments, in addition to increasing Vmax, low-glutamine growth caused a decrease in the apparent Km value; Vmax (mmole/1ICW.min) increased from 5.73 to 9.32 after growth in low-glutamine in expt. 1, and from 12.87 to 17.09 in expt. 2. Km (mM) decreased from 3.43 to 0.89 in expt. 1, and from 2.39 to 1.24 in expt. 2; (from s/v : s plots of data of 5min influx (v) at external glycine concentrations (s) 0.5-5mM). Because of the directions of change, results were taken as valid - see method.

To investigate whether efflux, as well as influx, was affected, normal and low-glutamine grown cells were loaded with radioactive glycine during 'lysis' (so that initial internal glycine* concentrations were equal) and efflux rates calculated from fractions lost per 2min time intervals in 'single-plate effluxes'. Fig. 26 shows the result from 1 experiment. Although influx was increased from 0.69 $\pm$ 0.06 to 1.28 $\pm$ 0.19 mmole/1ICW.min, in the same group of cells, efflux was not significantly altered after low-glutamine growth. There was a slight effect, in that half-times of efflux were 7min in control cells and 6min in low-glutamine cells. It appeared, therefore, that the influx but not efflux carrier system was modified. The resultant effect is seen in Fig.27 which shows the accumulation values and rates of equilibration of glycine* in normal and low-glutamine cells. Both HeLa and Xenopus cells grown in low concentration of amino acid had greater accumulative capacities than normal cells.

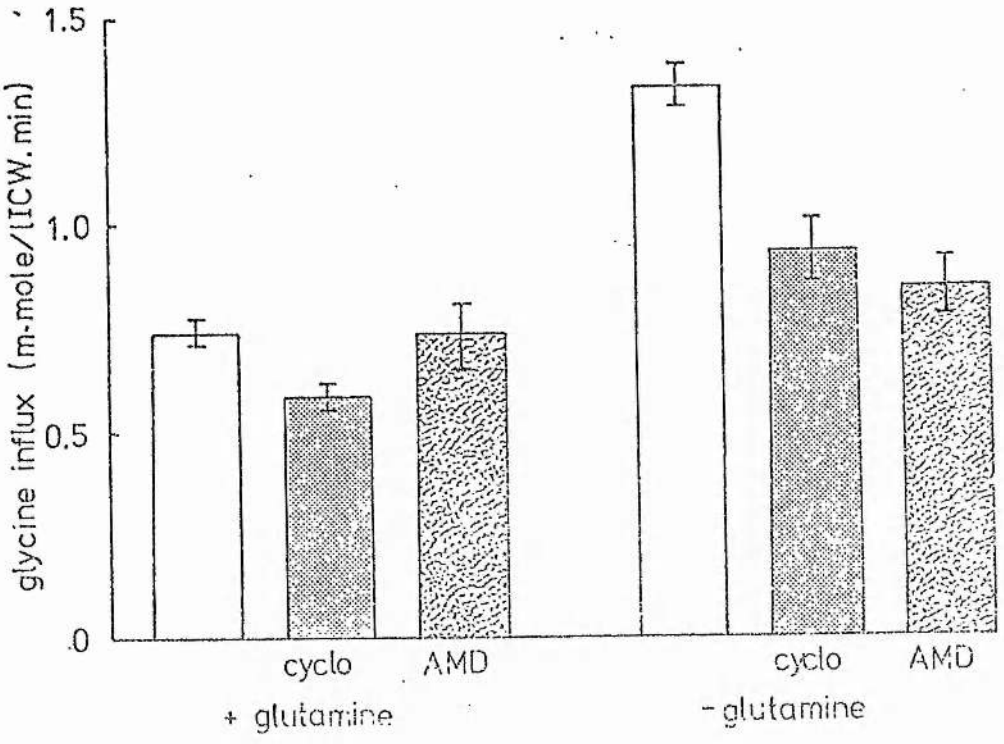


Fig.28.

Effect of protein synthesis inhibitors on glycine uptake, Xenopus

Cells grown for 3 days in normal growth medium. At time zero, medium changed to that indicated along the abscissa. Glycine influx measured from 2mM glycine* (26°C), in W&Q salt solution. Values at 7hr are given, as means of 4 readings ± SE bars. Cell number and volume not significantly altered within the time of the experiment.

It was of interest to determine how the cell was able to detect changes in the external amino acid concentration and how the resultant effect was mediated.

The development of the stimulation, or derepression, of uptake was, therefore, studied in the presence of the protein synthesis inhibitors cycloheximide (acting at the ribosomal level, to prevent the translation of mRNA) or actinomycin D (AMD), (acting to prevent the transcription of mRNA). Fig. 28 shows glycine influx measured 7hr after medium was changed to control or minus glutamine (open bars). The stippled bars show the effect of cycloheximide (3 μg/ml) and AMD (2.5 μg/ml), added with the change of medium. In cells grown in control medium, cycloheximide caused a significant (20%) reduction in glycine influx ($P < 0.01$). (In 1 further experiment, 6hr growth in medium + cycloheximide (15 μg/ml), caused a 33% decrease in glycine influx). No such effect was observed with AMD, ($P > 0.9$). After growth in 'low-glutamine' medium, influx was increased, ($P < 0.001$). AMD prevented the transport modification (with control, $P > 0.1$) but the block was not complete with cycloheximide, (with control, $P < 0.05$). In the same experiment, cells were treated with 'low-glutamine'/+ cycloheximide medium for 3hr and medium then changed to wash off the cycloheximide. The cells partially recovered from cycloheximide treatment within 8hr. Cells replaced into normal medium had influxes equal to those in control cells. Cells placed into medium + AMD, +/- glutamine, had uptakes equal to that in control cells.

Inhibitor studies, therefore, indicated that the efferent pathway of control was via protein synthesis. In addition, cycloheximide caused a reduction in normal uptake activity.

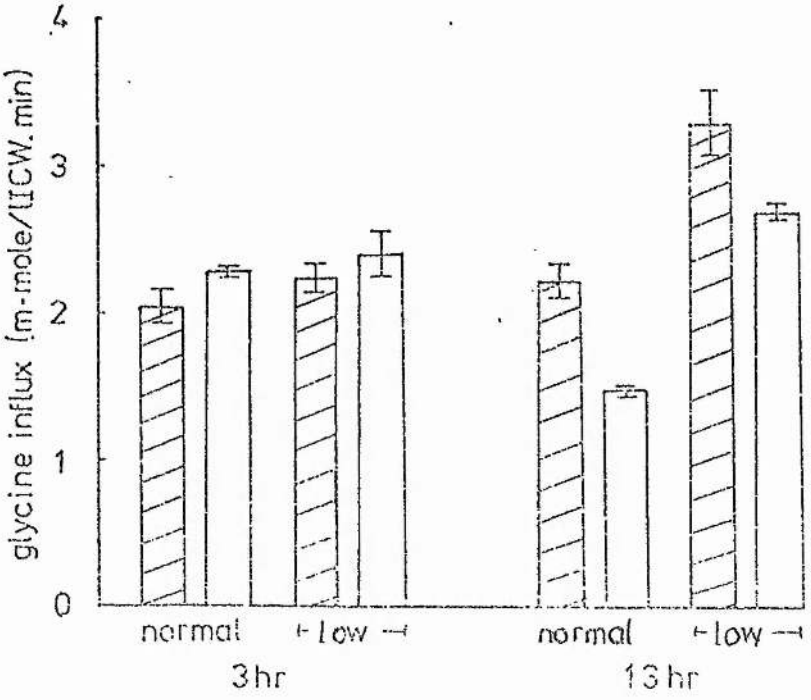


Fig.29.

Effect of growth in low-Na on (amino acid)$_e$ regulation of influx, HeLa

Cells grown 3 days in normal medium. At time zero, medium changed to +/- Na, or low-amino acid +/- Na. Normal Na was 140mM, (hatched bars); low-Na was 35-40mM, (open bars). Choline replaced Na. Glycine* influx measured over 5min (37°C) in normal Krebs, glycine 2mM. Cell number and volume not affected by treatment. Analysis of variance of 13hr values showed a significant increase of influx with low-amino acid growth ($P \lesssim 0.001$) and a significant decrease with low-Na growth ($P < 0.01$), with no significant interaction ($P > 0.5$). The difference between mean influxes into normal and low-amino acid treated cells was not significantly different from the difference between influxes into normal and low-amino acid cells, after low-Na treatment, ($P > 0.4$). Values are means of 2 readings, $\pm$ SE bars, (from 2 experiments).

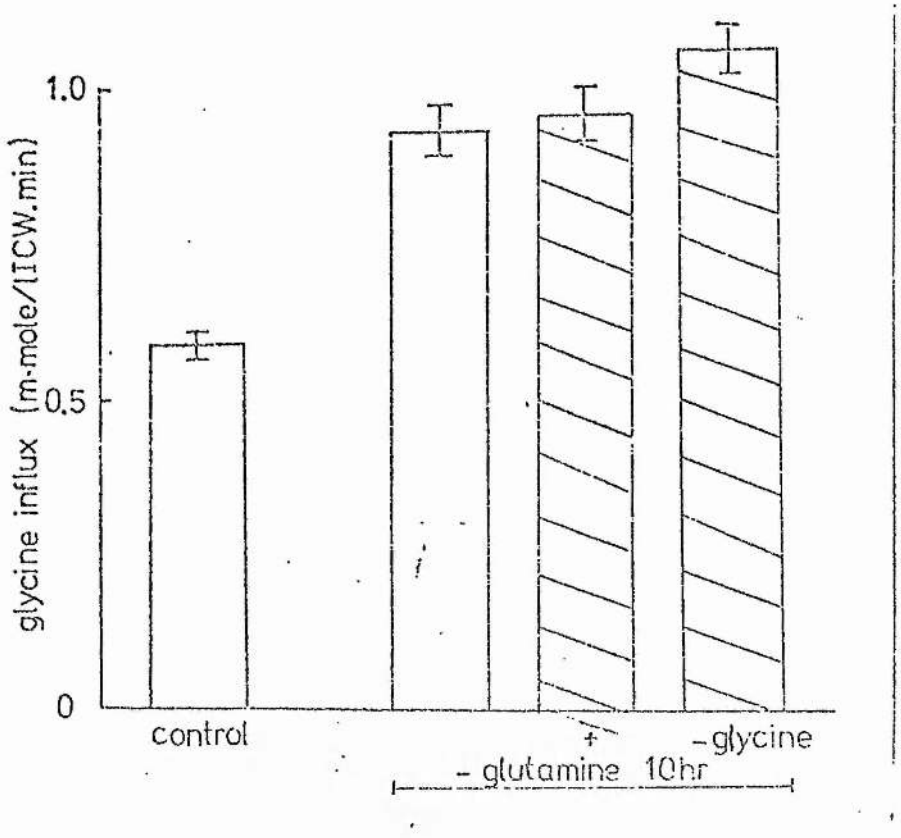


Fig.30.

Effect of 'lysis' on (amino acid) regulation of glycine influx, Xenopus

Medium changed to 'conditioned' -glutamine, at time zero. Cells 'lysed' (hatched bars) in the presence or absence of 20mM glycine, at 2hr intervals throughout the experiment. Glycine* influx subsequently measured, (5min, 26°C, 2mM glycine* in W&Q salt solution.) Control cells (open bars) incubated for 10min in W&Q before influx measurement. Cell volume reduced by 5% 7hr after -glutamine treatment begun; no significant effect of 'lysis', by analysis of variance. Cell number reduced by 20% in 'lysed'/ -glycine cells after 7-10hr. 1st column is the mean of 4 readings, remainder are means of 8 readings ± SE bars.

It was possible that the afferent pathway to control of transport (ie. the mechanism of detection of low external amino acid concentration) was mediated via a change in the free intracellular amino acid pool.

The intracellular amino acid concentration was therefore manipulated by (a) growth in low-Na medium, (b) 'lysis' and refilling, during the induction of the effect.

(a) The results of growth of HeLa cells in low-Na medium are shown in Fig. 29. 3hr after medium was changed, analysis of variance showed no significant effect of treatment; neither low-amino acid (1/5 normal amino acid mixture), ($P > 0.1$), nor low-Na, ($P > 0.1$). There was no significant effect between influx measured in control cells after 3 and 13hr of growth, (T-test, $P > 0.4$). After 13 hr growth in low-amino acid, glycine influx was increased when external Na was normal, and similarly in low-Na grown cells. Superimposed on this was a decreased influx, in both normal and low-amino acid cells, due to growth in low-Na.

(b) Fig. 30 shows the result from one experiment. Cells were 'lysed' repeatedly in the presence or absence of glycine (external concentration = 20mM) during growth in low-glutamine medium, so as to obtain cells with a varied intracellular pool and a reduced extracellular pool of amino acids. (T-tests showed no significant effect of 'lysis' at time zero). Analysis of all readings, however, showed a significant increase in glycine* influx with time in low-glutamine ($P < 0.001$), no effect of 'lysis' ($P > 0.05$) and no interaction, ($P > 0.1$). The increased influx, due to altered external amino acid concentration, occurred within 2hr, with no further significant increase, so that mean values measured during 2-10hr are given. T-tests of the last 3 columns showed no significant difference between control (non-'lysed') cells and cells 'lysed' in the presence of glycine. Cells 'lysed'

without glycine had a significantly greater influx (with control, $P < 0.05$, with 'lysed', plus glycine, $P < 0.025$). This represented a further 10% increase in influx.

In a separate, similar, experiment, loading with 20mM radioactively labelled external glycine during 'lysis' gave an initial internal glycine of 87mmole/lICW which had a half-time of loss of 33 min.

Both series of experiments suggested that the intracellular substrate concentration was not a major limiting factor in amino acid transport regulation.

DISCUSSION

Results indicated that glycine entered HeLa and Xenopus cells by a specific saturable process. The ability of the cells to accumulate glycine, above external concentrations, could be explained in terms of 'active' transport if the amino acid were considered as free inside the cell. Transport would then involve a specific 'carrier' system within the membrane, actively pumping glycine against a concentration gradient. An alternative hypothesis was that intracellular glycine was bound to some cellular constituent; the amino acid entering the cell by free diffusion, prior to binding. Evidence against this possibility has previously been presented:

(1) Preincubation of cells with amino acid should result in preloading of binding sites, so that experimentally measured influx of amino acid ought to be initially reduced. In Ehrlich cells, the opposite was found (Heinz, 1954) when glycine influx was actually increased by preincubation with glycine. This indicated that intracellular binding was not the rate-limiting step in uptake.

(2) Compartmentalisation of intracellular amino acid should result in a lag in exchange of bound fractions, compared with free glycine. The efflux coefficient of glycine from Ehrlich cells was found to be constant, (Heinz, 1957). In the experiments presented here, although there was no evidence for any fraction of cellular glycine exchanging with a delay as compared with the remainder, in either Xenopus or HeLa cells, the possibility of intracellular binding with a high turnover rate cannot be excluded.

(3) Simple binding was unlikely as neither Ehrlich cell fragments, (Heinz & Mariani, 1955), nor unannealed pigeon erythrocyte vesicles (prepared by sonication) (Lee, Beygu-Farber, & Vidaver, 1973), were able to bind or accumulate glycine.

Efflux from Xenopus cells was, like influx, saturable but the system had a much lower affinity for amino acid. It is assumed that the accumulative ability of the cell was due mainly to the difference in the Km values for entry and exit. (The possibility of intracellular compartments of high and low glycine was not considered and efflux values were calculated on the basis of total intracellular water.)

Two hypotheses concerning amino acid efflux have previously been suggested:

(1) that it occurs by passive back-diffusion, increasing proportionally with intracellular concentration, (Heinz, 1954: Scholefield, 1961). This, when considered in association with the saturable influx, explains the fall-off in equilibrium ratio observed when extracellular amino acid was increased.

(2) that the exit process is similar to that for mediated entry. Early work with Ehrlich cells demonstrated that leucine efflux deviated from linearity as intracellular concentration increased but the evidence presented for a saturable exit process was not conclusive as it was difficult to obtain sufficiently high intracellular concentrations (Oxender & Christensen, 1963). Approximate values for Vmax and Km for MeAIB, alanine, lysine and phenylalanine efflux have since been obtained, (Christensen & Handlogten, 1968). Values for Vmax were comparable to those for influx but Km values were much greater, (25x for MeAIB). Saturable efflux has also been demonstrated for alanine in rabbit ileum (Hajjar, Lamont, & Curran, 1970) and for glycine in mouse ascites tumour cells, (Eddy, Mulcahy, & Thomson, 1967). The latter workers noted that the kinetic constants which they obtained for efflux were similar to those expected for influx measured in reduced Na (14-5mM).

Results reported here support the latter hypothesis. Assumptions based on the former, however, would remain approximately correct, as the value for Km_{efflux} in Xenopus was considerably larger than the range of intracellular concentrations normally found (ie, the exit process was not normally saturated.)

Under normal conditions of transport measurement, no exchange diffusion could be demonstrated. The results are limited in that no investigations were carried out under conditions of reduced active transport and reduced carrier affinity, eg., in low-Na medium. If the contribution of exchange uptake to total influx were very small then the uptake might, under normal conditions, have appeared as a single system. Alternatively, exchange diffusion would not have been demonstrated if the substrate affinity of the active carrier were very great compared with that of the inactivated carrier.

Proposed mechanism of transport

The data obtained for equilibrium distribution-ratio was found to qualitatively fit a proposed mechanism of accumulative transport, based on a saturable carrier hypothesis, and Michaelis-Menten kinetics.

At the steady state (with no net transport) influx = efflux.

$$v_o = \frac{s_o}{Km^o + s_o} \cdot Vmax^o = v_i = \frac{s_i}{Km^i + s_i} \cdot Vmax^i$$

where:

v_o = influx
s_o = external glycine concentration
Km^o, $Vmax^o$ = kinetic constants of influx
v_i = efflux
s_i = internal glycine concentration
Km^i, $Vmax^i$ = kinetic constants of efflux

rearranging the equation:

$$\frac{Vmax^o}{Vmax^i} = \frac{(1 + Km^o/s_o)}{(1 + Km^i/s_i)}$$

Using the kinetic constants derived experimentally, not all the steady state glycine values quantitatively fit the proposed relationship between Vmax, Km, and glycine concentration (see Fig. 6). As all the necessary data were not derived from a single experiment, this may be due to interexperimental variation in carrier function rather than an invalid assumption.

General transport properties

The characteristics of the transport system were similar to those which have previously been used to identify non-electrolyte transport systems and provided evidence for the Na-K-gradient hypothesis.

Extracellular ion dependence

Reduction in extracellular Na resulted in a decreased influx of glycine and a decreased steady-state distribution ratio. In the absence of external Na, cells were unable to accumulate glycine against its concentration gradient. Extracellular Na could not be effectively replaced by K, choline, or Li ions. Kinetic analyses demonstrated that, in both cell types, the reduced influx of amino acid was associated with an increased apparent Km with no change in Vmax. This indicated that extracellular Na acted to increase the affinity of the carrier for substrate without altering carrier mobility. Data also fitted Michaelis-Menten kinetics if influx of glycine was correlated with extracellular Na at constant extracellular glycine concentrations. Results were consistent with a single Na ion being associated with the rate-limiting step in the influx of a single glycine molecule. There was no evidence to suggest that the carrier-complex formation involved the binding of 2 Na ions (a direct

relationship between amino acid influx and $(Na)^2$) as found by Vidaver (1964a) for glycine influx in pigeon erythrocytes and by Baker and Potashner (1971; 1973b) for glutamate influx in both crab nerve and squid axon.

Glycine influx was not dependent upon extracellular K. Results indicated that (1) high extracellular K was not inhibitory, (2) extracellular K was not an obligatory requirement for influx and (3) inactivation of the Na-K-pump, through lack of K, did not have an immediate effect on glycine influx.

Intracellular ion dependence

Intracellular ion levels were altered either directly by 'lysis' treatment, or indirectly by preincubation in low-K medium. (The latter treatment results in decreased activity of the Na-K-pump, and dissipation of the normally maintained ion gradients). Glycine influx into Xenopus cells was not affected by the variation in intracellular Na and K levels which could be obtained. In HeLa cells, however, 'lysis' experiments demonstrated that, as intracellular Na was raised, glycine influx was reduced. In this cell type the 'lysis' treatment, itself, caused a reduction in influx; so the effect of intracellular ion concentration on influx was investigated using low-" treatment. The 'lysis' results were confirmed in that glycine influx was reduced after preincubation in low-K media. In preliminary experiments it was not possible to distinguish whether this inhibition of transport was due to high intracellular Na, or high-Na plus low-K, as glycine influx could be correlated equally well with either Na or K concentration. In later work, sorbitol was used to iso-osmotically replace external Na to a variable degree; so that, after low-K treatment, cells were obtained with low-K plus high-Na or low-K plus normal Na. No correlation was then found between glycine influx and intracellular K, whereas influx

was proportional to intracellular Na. Variance about the correlation line was more equally distributed for a $1/v : f(Na)_i$ relationship, rather than a $v : f(Na)_i$ relationship. This indicated that the former relationship was more likely and, therefore, that there was not a finite concentration of intracellular Na at which glycine influx was zero. Decreased influx, due to increased intracellular Na, was associated primarily with a reduced Vmax. This suggested that, although extracellular Na altered carrier affinity for amino acid, the rate-limiting effect of intracellular Na on influx was via a change in carrier mobility.

Results for HeLa cells could, therefore, be explained in terms of the Na-gradient hypothesis. The sodium dependency of influx was consistent with the binding of a single Na ion per substrate carrier, so that the affinity of the carrier site for glycine was concomitantly increased. Intracellular Na decreased influx, possibly limiting the rate of return of carrier to the outer surface of the membrane. The apparent insensitivity of influx to intracellular ion levels in Xenopus cells may have reflected a cell-type variation in the relative affinities of intracellular Na and K for substrate carrier site. Thus the intracellular Na dependency did not necessarily preclude an effect of intracellular K in maintaining normal carrier turnover or reorientation.

The Na-gradient hypothesis predicts that amino acid efflux should be increased as intracellular Na is raised. In Xenopus cells, increasing intracellular Na up to 23mmole/l ICW (an increase which had no effect on influx) had no effect on rate constant of efflux. An increase of intracellular K above the normal level caused an accelerated efflux. This suggested that the efflux of K, down its transmembrane gradient, enhanced carrier orientation or that high concentrations of intracellular K altered carrier affinity for amino acid. Results would,

therefore, agree with the Na-K-gradient hypothesis.

Assuming that energy for transport was derived from both Na and K gradients, then the potential energy available was calculated from $2.3RT\log \left(\frac{Na_o}{Na_i} \cdot \frac{K_i}{K_o}\right)$ and compared with that required to maintain the observed steady state distribution ratios for glycine, calculated from $2.3RT\log \left(\frac{glycine_i}{glycine_o}\right)$. Sufficient energy was available in both HeLa and Xenopus cells. Using steady state data obtained under normal conditions of transport (see figs 4, 5, 6) the maximum energy requirement for glycine accumulation in Xenopus cells was approximately 2000 cal/mole compared with a potential energy of approximately 2800 cal/mole (a required efficiency of 71%). In HeLa cells, the requirements at 2mM external glycine was 2275 cal/mole, compared with an available potential energy of 3482 cal/mole, (efficiency = 65%).

Action of metabolic inhibitors and ouabain

No evidence was obtained for any direct link between cellular metabolism and initial rate of glycine uptake in either cell type. In HeLa cells, the accumulative capacity for glycine was less after ouabain treatment if sufficient time were allowed for alteration in intracellular ion distribution. In agreement with low-K results, the observed increase in intracellular Na resulted in a decreased Vmax of influx without a variation in Km. In Xenopus cells, accumulation was less after ouabain treatment, possibly due to an effect on efflux rather than influx as prior findings predicted that the variation in intracellular ions was insufficient to affect influx or limit accumulation through lack of potential energy.

Thus the detrimental effect of metabolic inhibitors and ouabain on steady state distribution ratio could

be explained, in HeLa cells, in terms of altered alkali-metal ion distribution and the subsequent effect on influx. This was unlikely to be the case in Xenopus cells. A direct effect of ouabain on efflux was not investigated but cannot be excluded.

Effect of 'fresh' and 'conditioned' medium

If growth medium was replaced by 'fresh', but not 'conditioned', medium, glycine influx was reduced in both Xenopus and HeLa cells. The half-time of decay of influx activity was similar to that found when low-glutamine cells were replaced into normal 'conditioned' medium. The reduction in transport did not involve protein synthesis and kinetic analysis showed that the effect was associated with a reduced Vmax and no change in the apparent Km. This suggested that although the structure and affinity for glycine of the carriers remained unaltered, there was a reduction in either the rate of carrier turnover or number of active carriers. The results presented were consistent with inhibition of synthesis of transport carriers.

Since the original observation, the work has been extended in this laboratory. Similar results have been found for Rb^{*} and 2-Deoxyglucose, but not chloride, influx, in Py3T3 cells, (Brown, Hume, Lamb, & Weingart, 1974). It is suggested that the cells release into their culture medium a dialysable, low molecular weight substance, whose absence causes a reduced transport activity.

Different half-times of decay of activity may represent differences in the normal rate of turnover of the sites, or different half-lives of the carrier proteins which themselves are in a dynamic state of turnover.

Effect of serum concentration during growth

Xenopus and HeLa cells responded to the serum concentration of their growth medium by regulating their glycine transport function. A progressive increase in concentration of either foetal-bovine or calf serum, during growth, resulted in a proportional increase in experimental influx and accumulation of glycine.

The altered capacity of the system was characterised by a variable Vmax with no change in the apparent Km of influx. This suggested that the regulatory mechanism was via control of the number or rate of turnover, of exposed, functionally active sites, rather than an altered carrier structure. Qualitatively similar alteration of kinetic constants have been reported for serum stimulation of uridine uptake in mouse and hamster embryo cells, previously grown in serum-free medium, (Hare, 1972b; Lemkin & Hare, 1973).

It was possible that high serum concentrations acted to potentiate glycine transport (a) by counteraction of a transport inhibitor, (b) by induction of synthesis of additional, identical sites, or (c) by modification of pre-existing sites.

The development of the serum dependence was slow (48hr) and indicated a time-dependent modification of the cells, serum or culture medium. Considering the latter possibility, confluent 3T3 cells have been shown to release an inhibitor which decreased the uptake of certain RNA precursors and whose action was counteracted by serum, (Pariser & Cunningham, 1971). Hare has reported the appearance of inhibitor of uridine transport in medium which had supported mouse embryo cells for 24 to 72hr, (Hare, 1972b). Because of the observed 'conditioning effect' in Xenopus and HeLa, it seemed improbable that a similar inhibitor for glycine transport existed. It was, however, possible that the growing cells released a substance which increased the serum-sensitivity of the cells. Evidence has been cited for the release of serum-

sensitising factor by confluent BHK_{21} cells, which was active on non-confluent cells, enhancing the DNA synthesis response of the cells to added serum, (Clarke & Stoker, 1971). The existence of a similar factor in HeLa and Xenopus cells would explain the time-lag before the stimulatory effect of serum could be observed. It is postulated that the latter effect is due to the presence of active factors which were able, directly or indirectly, to act as stimulators of glycine transport. No attempt has been made to identify these serum constituents.

Serum has previously been reported to stimulate amino acid transport. An increased serum concentration (from 5 to 20%) in growth medium increased leucine transport in both pre- and post-confluent cultures of MRC-5 cells (a diploid line from human foetal lung tissue) although the effect was only slight, (Griffiths, 1972). If 10% serum was included during a 4hr incubation of embryonic chick bone with AIB, then the uptake of the amino acid was increased, (Adamson & Anast, 1966). In the latter case, the authors noted a similar serum effect on proline incorporation and suggested that a non-dialysable serum factor stimulated amino acid incorporation through its stimulation of amino acid transport.

Using the protein synthesis inhibitor, cycloheximide, evidence was obtained, (see Fig. 21), in support of a labile transport carrier protein, (see later discussion of low-amino acid treatment). In addition, inhibitor studies showed that protein synthesis was necessary for the serum-dependent increase in transport capacity. Because of the non-specific action of cycloheximide it was not possible, however, to conclude whether the lack of acquisition of transport was due to lack of synthesis of an essential carrier protein; for instance, it may reflect a lack of serum-sensitising protein, or cycloheximide may have prevented the modification of pre-existing transport sites.

It is not known whether the observed stimulatory effect of serum on glycine transport reflects a general action on membrane transport systems, nor whether it is integrally associated with the growth-promoting activity of serum.

Regulation of transport by external amino acid concentration

In both Xenopus and HeLa cells, the concentration of amino acid in the growth medium was demonstrated as regulatory in the control of transport of glycine, (an A-mediated amino acid). As the concentration of similarly transported amino acids was increased, the transport activity, represented experimentally by glycine influx, decreased to a minimum value at about the normal growth concentration. Glycine influx was not influenced by the prior growth concentration of leucine, (an L-mediated amino acid, transported by a system of exchange rather than active transport).

Results were thus similar to those obtained by Gazzola but show a varying concentration dependence, rather than an 'all-or-none' effect, (Gazzola et al., 1972).

Control of transport was primarily through a variation in Vmax of uptake, indicating a change in either carrier number or activity so that more carrier proteins, of the same type, were operating, per unit of time, in low amino acid-grown cells. In HeLa cells, reduction of external concentration of transportable substrate also resulted in a lower value for Km; that is, a higher carrier affinity for glycine. This suggested a modification of carrier structure but may have been due to a change in the Na-sensitivity of the carrier.

The efflux rate of glycine was not significantly altered by the external concentration of amino acids during growth. Consequently, cells grown in lower concentrations of amino acids had a greater

accumulative capacity than normal cells. It is suggested that this allowed the maintenance of more normal intracellular amino acid pools despite reduced extracellular concentrations. Up to 24hr after a deficiency of 1 amino acid, HeLa cells have been shown to be able to maintain their free intracellular pools with little change, excepting that the amino acid missing from the medium was missing from the cell pool, (Piez & Eagle, 1958). This presumably occurred despite a variation in the competitory interaction of actively transported amino acids.

The development of the stimulation, or derepression, of uptake was studied in the presence of protein synthesis inhibitors. Actinomycin D (AMD) was used to prevent transcription of mRNAs on their DNA templates (Hamelin, Larsen, & Tavitian, 1973) and cycloheximide (affecting the process of peptide bond formation, or 'transl__ation') used to inhibit protein synthesis, (Grollman, & Mon Tuan Huang, 1973).

A decay in normal influx activity was observed during cycloheximide treatment; an effect previously observed during serum studies. It appeared that continuous synthesis of protein was necessary to maintain normal transport function. The results are consistent with the view that membrane components directly involved in carrier-mediated active transport were in a dynamic state of turnover and that the decay observed was due to protein catabolism. AMD had no effect within the 7hr of the experiment, indicating a reserve store of the appropriate mRNAs. Simultaneous synthesis and degradation of protein would normally be matched and no overall effect observed. It has been suggested that the significance of dynamic, rather than static, equilibrium may lie in the fact that it is easier to stimulate to net synthesis or degradation, (Pasternak, 1973).

Frizzel has suggested a turnover of alanine carrier in the brush border of rabbit ileum, (Frizzel,

Nellans, Acheson & Schultz, 1973). An injection of cycloheximide, 3hr prior to sacrifice, led to a 59% reduction in alanine influx (a 69% reduction in Vmax) across this tissue. Hare has used inhibitor studies to provide evidence for a labile, transport-active protein for uridine transport in mouse embryo cells, (Hare, 1972a,b). Similarly, evidence was obtained for the existence of a protein (or proteins) with a short half-life, concerned with AIB transport across intact rat diaphragms, (Elsas, Albrecht, & Rosenberg, 1968). In this latter preparation, evidence was given to show that the cycloheximide effect was a true decay of activity rather than merely a reflection of generalised cellular disfunction.

Both cycloheximide and AMD prevented the acquisition of additional transport activity. The incomplete inhibition with cycloheximide may represent time delay in the action of the antibiotic; possibly membrane-limited transport, as the protein synthesis block has been reported to be immediate, (Grollman et al., 1973).

As cycloheximide has a reversible action on protein synthesis (Grollman, et al.,1973), cells can be incubated with cycloheximide in a condition of inhibited mRNA translation but allowed mRNA transcription. This technique was successfully used in cardiac cell suspensions to demonstrate that external amino acid control of influx was at the level of mRNA transcription rather than translocation, (Franchi-Gazzola et al., 1973). The results presented here, however, showed that after "low-glutamine/+ cycloheximide" treatment, removal of cycloheximide, although preventing further decay of activity, did not result in a subsequent increase in glycine uptake. They differ from those of the Italian workers in that there was no evidence for stimulation of specific mRNA transcription during cycloheximide preincubation. It is suggested that protein synthesis was required for the initial regulatory signal although subsequent control may be at the level of transcription.

Genetic feed-back control. Results may represent a genetic regulation of carrier protein, analogous to that found for enzyme synthesis in bacteria. The latter is controlled by (a) structural genes, and (b) regulatory genes governing the expression of (a). For example, the synthesis of β-galactosidase in E. coli is controlled by a region of the genome which contains 4 loci; 1 contains the code for amino acid sequence and 2 are regulatory, (Vogel, 1961). Regulatory genes are under repressor action. Repressors are generally specific, end-products of pathways containing the repressible enxymes. They act so as to match synthesis to requirement of end-product by (a) 'end-product inhibition' (occurring at the level of enzyme action, rather than formation) (b) 'repression' of enzyme synthesis (a slower control mechanism depending upon dilution of pre-existing enzymes by growth), (Umbarger, 1961; Wijesundera, & Woods, 1960). Derepression would represent de novo synthesis of enzyme protein (Vogel, 1961) and repression/derepression would depend on the intra-cellular repressor concentration.

A feedback control has been reported in HeLa cells for synthesis of glutamyl transferase (which catalyses the reaction glutamic acid to glutamine) with glutamine as the repressor, (DeMars, 1958). More recently, Barnes has shown that the omission of glutamine from the growth medium caused an increase in the activity of the same enzyme in L-cells, (Barnes, Youngberg, & Kitos, 1971). If cells were then refed with glutamine-sufficient medium, enzyme activity decreased within 24hr to less than the starting value, indicating that glutamine acted to regulate the level of activity. If repressor action is similarly the principal regulatory device in carrier synthesis, then the control of glycine transport demonstrated here would reflect 'repression' of carrier synthesis at high external substrate concentrations rather than the immediate 'end-product inhibition' of carrier activity.

A non-genetic feedback control (in addition to control at the level of protein synthesis) has been reported for methionine transport in the fungus, Neurospora crassa, (Pall, 1971). Uptake was regulated directly by the internal amino acid concentration by the mechanism of 'transinhibition'. The binding of amino acid on the active site at the inner side of the membrane led to inhibition of methionine transport activity. This was unlikely to be of importance in Xenopus and HeLa cells as neither exchange diffusion nor transinhibition could be demonstrated.

The mechanism by which the labile carrier system received a regulatory input from the extracellular environment was investigated. It was possible that the afferent pathway to control was through a variation in quantity of some metabolite of glutamine or the level of a specific enzyme, itself regulated by glutamine concentration. This seemed improbable because of the ability of non-metabolisable AIB to substitute for glutamine in causing repression of glycine uptake. False feedback, however, has been reported for bacterial enzyme synthesis; an analogue substituting for an end-product in controlling enzyme function, (Umbarger, 1961).

An alternative mechanism was via detection of a change in the size of the intracellular amino acid pool. The latter is in dynamic equilibrium with that of the medium and depletion of any one of the essential amino acids results in the disappearance of that amino acid intracellularly, structural changes within 12-24hr and eventual cell death, (Cohen, Nylen, & Scott, 1961; Eagle & Piez, 1962). Glutamine is included in the 13 essential amino acids being a precursor for the synthesis of glutamic acid and asparagine, (Eagle & Piez, 1962). Under exceptional circumstances, cell death can be avoided. If HeLa cells were grown in high, non-physiological, concentrations of the non-essential amino acid, glutamic acid, and then starved

of glutamine, the cells survived due to a rise in glutamyl transferase activity, (DeMars, 1958). Barnes has suggested that, although increasing glutamate in glutamine-deficient medium favoured L-cell growth, the effect was not due to an increase in glutamyl transferase, (Barnes et al., 1971). Given altered growth conditions, however, cells were, in some manner, able to adapt to assure their survival.

The findings in the studies reported here, that the cells survived despite glutamine deprivation, may be due to a small concentration of glutamine present in the serum. Because of its associated enzymes, serum undergoes proteolysis to give free amino acids (Eagle & Piez, 1961). This, itself, may have sufficed for protein synthesis as only small concentrations of essential amino acids are required. In HeLa cells, intracellular valine had to be only 2-3 times a critical minimum concentration of 0.02mM, to give a maximum level of growth rate and protein synthesis, (Eagle & Piez, 1962). Minimal effective external amino acid concentrations were 0.0015-0.006mM, (Eagle & Piez, 1961). That transport was found to be proportional to external concentrations far in excess of these values suggested that, although protein synthesis may be regulated by the influx of precursors (Adamson, Herington, & Bornstein, 1972; Phang, Finerman, Singh, Rosenberg & Berman, 1971), the rate of protein synthesis was not the limiting factor in transport regulation.

If the limiting factor were intracellular pool size, then direct manipulation of the latter, during low amino acid-growth or reduction in pool size by incubation in low Na, ought to affect the regulatory mechanism. In HeLa cells, low-Na treatment resulted in a partial inhibition of derepression of influx; a finding similar to that reported in chick cells, (Gazzola et al., 1973). 3hr preincubation of rat uterus in Na-free Krebs-Ringer bicarbonate buffer

resulted in a decreased AIB uptake activity, (Riggs & Pan, 1972). This apparently contradictory finding may be an 'external Na concentration' effect, similar to that noted for HeLa and Xenopus cells. It was expected that a reduction in external Na would influence the external amino acid regulation by causing a further decrease in intracellular pool and a further stimulation, or derepression of uptake. In fact, it caused a reduction in influx, compared with that measured after normal Na incubation, in both control and low amino acid grown cells. Because of this superimposed effect, results were difficult to interpret. It appeared, however, that the intracellular pool size did not play a major part in the afferent control pathway, the external amino acid derepression effect being about equal with both normal and low-Na incubation.

Supporting evidence was obtained by varying intracellular amino acid pool size by 'lysis' during incubation of Xenopus cells in low-glutamine medium. Refilling cells with glycine did not prevent the derepression of uptake caused by low external amino acid concentration. Repeated 'lysis', in the absence of amino acid, however, resulted in a further small but significant increase. This was possibly due to a minor contribution to control, via a very much reduced intracellular pool.

One further possible limiting factor to control, was the rate of membrane transport of amino acids. It is noted that external Na caused a reduction in influx, in both types of cell, through an altered Km, or carrier affinity; Vmax was not affected. Because of the external Na effect on regulation of transport activity, it is suggested that substrate control was via the rate of carrier activity per unit time, rather than the quantity of substrate transported per unit time. Any external amino acid competing for that transport site could then act as a regulatory substrate.

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Effect of growth in various concentrations of amino acids on the properties of the A-mediated amino acid uptake system in cultured cells

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The present experiments were designed to investigate whether the amino acid uptake system of HeLa and *Xenopus laevis* kidney cells could adapt to altered environmental conditions.

Fig. 1 shows that the [^{3}H]glycine influx into HeLa cells varies inversely with the amino acid concentration in the growth medium. This has the following characteristics:

(1) Increased glycine uptake activity is prevented by growth in mixtures of the A-group amino acids (Oxender & Christensen, 1963), by glutamine, or by α-amino-isobutyric acid, and so is not specific to one amino acid and is not due to the metabolic products of the amino acids.

(2) The time course of the effect is rapid. A change in initial velocity can be detected in 2 hr and is complete within 10 hr.

(3) Cellular Na and K concentrations are unaltered by changing the amino acid concentration of the growth media, so that the effect is not mediated in this way.

(4) The effect is blocked by concentrations of cycloheximide which block protein synthesis (15 μg/ml.).

(5) The change in the uptake carrier system is associated with an increase in V_{max} [HeLa and *Xenopus*] and a decrease in the apparent K_m [HeLa only].

(6) Cells 'lysed' and reconstituted (Lamb & Lindsay, 1971) showed a similar influx pattern to Fig. 1. Control and treated cells refilled with 69 mM [^{3}H]glycine* had the same efflux.

It is concluded that both HeLa and *Xenopus laevis* kidney cells can alter their A-mediated amino acid uptake system in response to alterations in the amino acid concentration of their growth medium. These conclusions are similar to those recently reached by Franchi-Gazzola, Gazzola, Ronchi, Saibene & Guidotti (1973).

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[P.T.O.

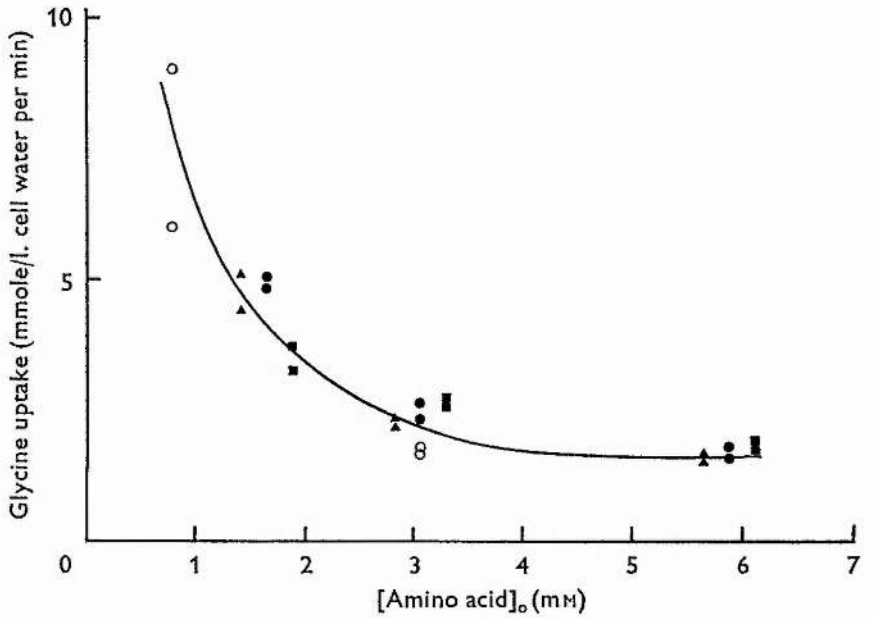

Fig. 1. Effect of growth in various amino acid concentrations on subsequent [^{3}H]glycine uptake. HeLa cells (clone S 3) were grown for 3 days in the concentrations of A-mediated amino acids shown and then the uptake of [^{3}H]glycine* measured over a 5 min period in Krebs solution containing 1% serum and 2 mM glycine*. The glycine* uptake is altered by the growth conditions. Data from 2 experiments (open and filled symbols) with 1% (▲), 10% (●, ○) or 20% (■) serum. The concentration of A-mediated amino acids in normal growth media is about 3 mM.

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An effect of conditioned growth media on transport systems of cultured cells

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In the course of experiments on amino acid uptake by HeLa and *Xenopus laevis* kidney cells (Hume & Lamb, 1974) we observed a reduction in the glycine influx after the medium in which cells were growing ('conditioned medium') was replaced by fresh medium. Further experiments, using Py3T3 cells, have shown that this procedure also reduces the influx of Rb^+ and 2-deoxyglucose but not the influx of chloride. In all cases the 'conditioning effect' was a 30–40 % reduction in influx. The time course of the effect was varied.

The reduction of glycine influx into HeLa cells persisted fully for at least 6 hr. Recovery to the normal conditioned level was almost complete after 36 hr. Kinetic experiments, carried out after 92 hr treatment with fresh medium, suggest that the reduced influx arises from a decreased V_{max} with no change in the apparent K_m of the glycine transport system. When Py3T3 cells were replaced in conditioned medium after 30 min exposure to fresh medium the glycine influx showed a rapid recovery (10 min) to the normal conditioned level. Changing cells from conditioned to conditioned medium or from fresh to fresh medium produced no sustained effect.

Measurements of the Rb^+ influx into Py3T3 cells have shown that the conditioned medium can be stored for at least 3 days at 4° C without losing the ability to sustain the higher level of transport. Conditioned medium dialysed against Krebs for 24 hr caused a reduction of the influx similar to that caused by the addition of fresh medium. A high molecular-weight fraction from conditioned medium (separated by rotary film evaporation at 35° C) did not alter the property of fresh medium.

We tentatively conclude that cells produce a dialysable substance which is released into the growth medium. The absence of this substance from the cell's bathing medium causes reduced activity of several transport systems in the cell membrane.

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